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IGF AVAILABILITY
DURING BONE DEVELOPMENT
IN FOETAL SHEEP

A thesis

submitted in fulfilment

of the requirements for the Degree

of

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at the

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by

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ABSTRACT

Skeletal growth is achieved through endochondral ossification at the growth plate and intramembranous ossification at the bone surface. Evidence suggests that the insulin-like growth factors, IGF-I and IGF-II, are major factors involved in these processes.

The objective of this thesis was to identify the potential role IGFs play in the growth and development of foetal sheep long bones. To achieve this, the localisation of IGF-I, IGF-II and IGF BP proteins was determined by immunohistochemistry in long bones isolated from foetal sheep between 50 and 140 days gestation. IGF-I and IGF-II binding sites were localised in bone by radioligand binding, and further characterised using an affinity cross-linking assay. The local production of IGF-I, IGF-II and IGF BP-2, -4 and -5 was analysed by *in situ* hybridisation and Northern blot hybridisation. To investigate whether circulating IGF-I could contribute to the pool of IGF-I in bone, labelled IGF-I was infused into the circulation of foetal sheep *in utero* and the localisation of labelled IGF-I was analysed in long bones.

The results demonstrated that IGF-I, IGF-II and IGF BP proteins were similarly localised during foetal development in many areas of bone. In the hypertrophic zone of the growth plate, the presence of IGF-I, IGF-II and IGF BPs in chondrocytes coincided with specific cellular binding of IGF-I and IGF-II, which was found to be to the Type I and Type II IGF receptors, respectively. Additional binding of IGF-I and IGF-II was found in cartilage matrix, which was likely to be to extracellular matrix proteins. In bone, IGF-I, IGF-II and IGF BPs were not only found in cells from the bone cell lineage, but were also detected in bone matrix.

IGF-II appeared to be locally produced during foetal development in the growth plate and diaphysis, from multiple IGF-II transcripts sized between 1.0 and 6.4 kb. At the cellular level, IGF-II mRNA was abundant in those cells where IGF-II peptide was located. Although IGF-I mRNA (7.4 kb) and IGF-I peptide were also co-localised, levels of IGF-I mRNA were barely detectable in foetal bone at any age. There was no evidence of local production of IGF BP-2, -4 or -5 in the growth plate. Labelled IGF-I derived from the circulation was available to a

limited number of cells in bone, located at the boundary between the growth plate and metaphysis, and within vascular canals embedded in the growth plate.

Overall, results from this study demonstrate that IGF components are widely and similarly distributed in long bones during foetal sheep development. However, the availability of IGF-I and IGF-II to cells in bone appear to be different. While the local production of IGF-II was likely to produce effects on many cells in bone, neither the level of IGF-I expression nor the presence of circulating IGF-I was likely to contribute sufficient levels of IGF-I to enable cells in most areas of bone to respond to IGF-I actions. Sufficient levels of IGF-I appeared to be available in only one area, located at the boundary between the growth plate and bone. This area has recently been identified as an important regulatory region for endochondral ossification.

Dedicated to

Rona Hodges

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LIST OF ABBREVIATIONS

The International System of Units (S.I.) was used for all weights and measures.

ABC complex	avidin-biotin peroxidase complex
ATP	adenosine-5′-triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
CTP	cytidine-5′-triphosphate
dATP	2′deoxyadenosine-5′-triphosphate
dCTP	2′deoxycytidine-5′-triphosphate
dGTP	2′deoxyguanosine-5′-triphosphate
dTTP	2′deoxythymidine-5′-triphosphate
DNA	deoxyribonucleic acid
DAB	diaminobenzidine
dpm	disintegrations per minute
DSS	dissucinimidyl suberate
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid disodium salt
GH	growth hormone
GTP	guanosine-5′-triphosphate
HEPES	[4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid]
IgG	immunoglobulin G
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IGF BP	insulin-like growth factor binding protein
LB	Luria -Bertani bacterial broth
M	mole
mRNA	messenger ribonucleic acid
MOPS	(N-morpholine)-propane-sulphonic acid
NEM	N-ethylmaleimide
PMSF	phenyl methyl sulphonyl fluoride
PBS	phosphate-buffered saline
rpm	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
S.E.M	standard error of the mean
Tm	temperature at which half the hybrid molecules become single stranded
TEMED	N,N,N,N-tetramethyl ethylenediamine
tRNA	transfer ribonucleic acid
UTP	uridine-5′-triphosphate
v/v	percent "weight in weight". Expresses the number of grams of an active constituent in 100 grams of a solution or mixture.
w/v	percent "weight in volume". Expresses the number of grams of an active ingredient in 100 ml solution.

CHAPTER 1

INTRODUCTION

1.1 LONG BONE GROWTH AND DEVELOPMENT

Long bone formation begins in a vertebrate during the embryonic period with limb bud outgrowth. From this, individual bones develop, grow and remodel throughout an individuals life. The chondrocyte, osteoblast and osteoclast cells and their precursors are the principal cells involved in bone growth and development. Successful development of these cell lineages to form bone also relies on the development of an appropriate vascular system.

The process of bone development, growth and remodelling is coordinated by a large number of systemic and local factors which control the timing of differentiation and cell division in particular cell lineages. IGF-I and IGF-II are key factors known to be associated with long bone growth and development in many species. The IGF system in sheep long bone growth and development is the focus of the current study.

1.1.1 Cell Lineages Involved in Bone Growth

1.1.1.1 **Osteogenic and Chondrogenic Cells**

It is believed that bone and cartilage cell lineages are derived from mesenchymal stem cells (Aubin *et al.*, 1993). Mesenchymal cells differentiate to form committed osteogenic and chondrogenic progenitor cells in addition to progenitor cells for other cell lineages such as

myoblasts, adipocytes and fibroblasts (Figure 1.1) (Aubin *et al.*, 1993). Osteogenic cells reside in the inner layer of the periosteum and the endosteum which line the internal surfaces of all cavities within bone (Ham and Cormack, 1979; Owen, 1963). Chondrogenic cells are found in the inner layer of the perichondrium (Ham and Cormack, 1979). The periosteum and perichondrium form a continuous membrane layer on the outside of bones. Osteogenic and chondrogenic cells are spindle-shaped cells with oval or elongated nuclei (Figure 1.1) (Scott, 1967).

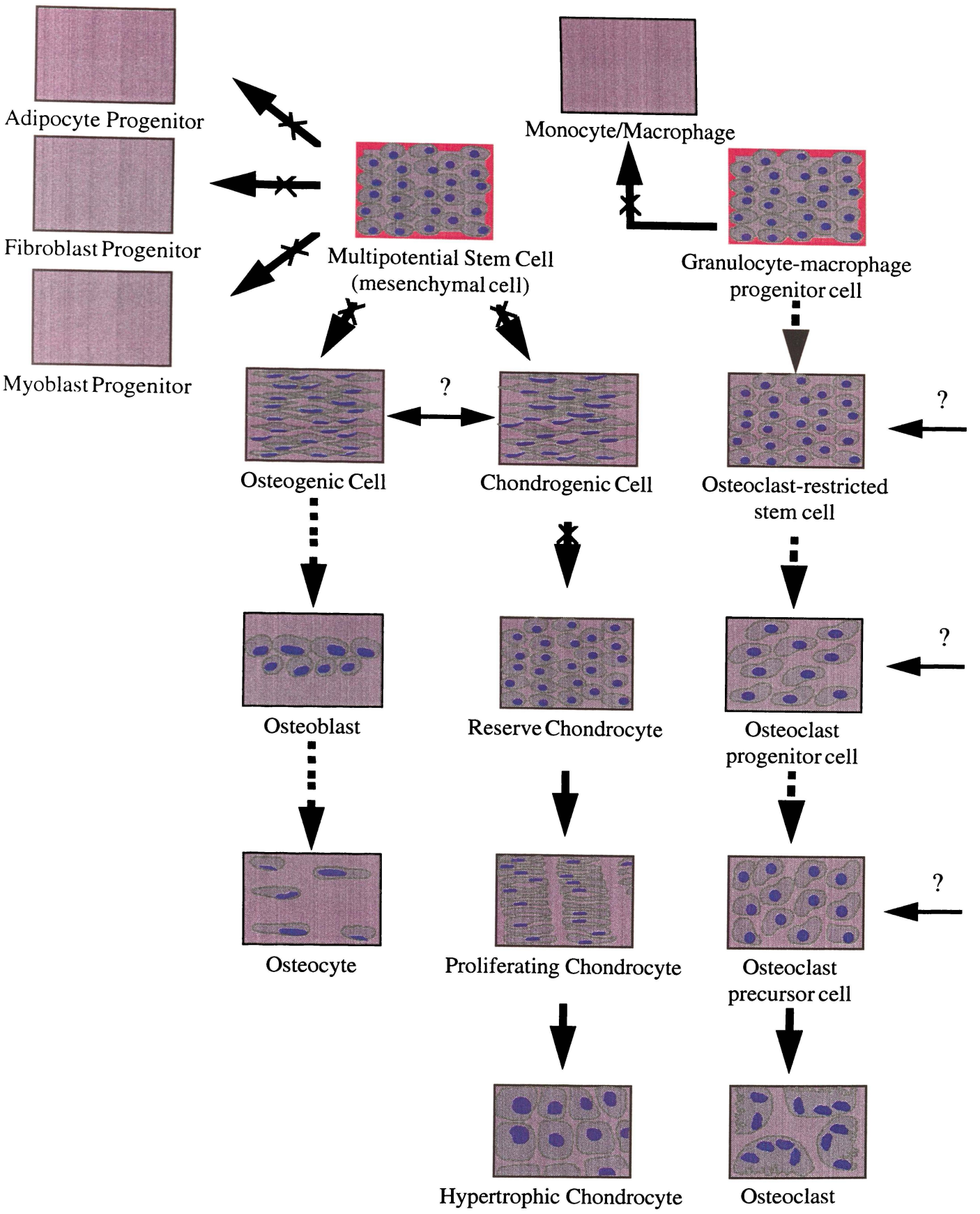
Most evidence suggests osteogenic and chondrogenic cells are identical pluripotent stem cells (Owen, 1988; Aubin *et al.*, 1993) since they retain their capacity to divide throughout life and it is possible to trace bone and cartilage formation to these cells. Their potential to differentiate along a particular pathway differs due to the location in which they are found. Location in either the periosteum or perichondrium determines the microenvironment they will be exposed to and hence the pathway down which they will differentiate (Ham and Cormack, 1979). If the cells are in a vascularised area they will differentiate into bone cells. In an avascular area they differentiate into cartilage cells.

1.1.1.2 **Cartilage Cell Lineage**

Chondrogenic cells differentiate into chondrocytes (Figure 1.1). Chondrocytes in the epiphyseal growth plate pass through defined morphological stages of maturation (Howlett, 1979). Initially chondrocytes are in a 'resting' phase where little cell division occurs (Robertson, 1990; Kember, 1960), but they subsequently enter a phase of proliferation characterised by flattened cells in a stacked arrangement (Howlett, 1979). Following this, chondrocytes mature into hypertrophic chondrocytes which increase dramatically in size due to glycogen uptake and storage. Corresponding with changes in cell appearance, there are changes in cartilage matrix composition and nutrient availability during maturation (Robertson, 1990). The cartilage matrix surrounding hypertrophic chondrocytes calcifies, thus posing a diffusion barrier to nutrients (Robertson, 1990), which it is believed is a factor in chondrocyte death and the subsequent replacement of cartilage with bone (Caplan, 1988).

Figure 1.1
Differentiation of Bone Cell Lineages
Postulated steps of differentiation and maturation in osteoblast, chondrocyte and osteoclast cell lineage's. As a cell proceeds through its lifecycle, there is increasing differentiation and maturation and decreasing proliferative capacity.
A red cross on the pathway denotes differentiation.
A broken arrow indicates that various cells with distinguishable phenotype and degree of maturity have been identified at this stage of the pathway.
A question mark represents steps in the pathway that have yet to be fully characterised.

Source:
Drawn from information contained in Aubin, Turksen and Heersch (1993) and Burger and Nijweide (1991)


Adipocyte Progenitor
Fibroblast Progenitor
Myoblast Progenitor
Multipotential Stem Cell
(mesenchymal cell)
Monocyte/Macrophage
Granulocyte-macrophage
progenitor cell
?
Osteogenic Cell
Chondrogenic Cell
Osteoclast-restricted
stem cell
?
Osteoblast
Reserve Chondrocyte
Osteoclast
progenitor cell
?
Osteocyte
Proliferating Chondrocyte
Osteoclast
precursor cell
?
Hypertrophic Chondrocyte
Osteoclast
Bone Formation
Cell Lineage
Cartilage
Cell Lineage
Bone Absorption
Cell Lineage

1.1.1.3 Bone Cell Lineage

Based on morphological and histological studies, bone cells are categorised in a presumed linear sequence from osteogenic cells to osteoblasts and then to osteocytes (Figure 1.1) (Aubin *et al.*, 1993; Scott, 1967; Owen, 1967). Osteogenic cells differentiate into osteoblasts which synthesise and secrete an organic extracellular bone matrix on existing bone surfaces (Rodan and Rodan, 1983; Ham and Cormack, 1979). Active osteoblasts are cuboidal, polar, basophilic cells lining the bone matrix at sites of active matrix formation (Nijweide *et al.*, 1986). Inactive osteoblasts called bone lining cells are flat and highly elongated cells with spindle-shaped nuclei (Menton, 1984). Once surrounded by bone matrix, calcification of the matrix occurs and osteoblasts differentiate into osteocytes. These are the most terminally differentiated cells of the bone cell lineage. Although osteocytes are completely surrounded by bone matrix, communication and nutrition are possible via thin canaliculi connections to other osteocytes, osteoblasts and nearby capillaries (Marks and Popoff, 1988). Osteocytes are thought to play a role in preserving the structure of the bone matrix they inhabit and may have limited matrix absorption functions (Ham and Cormack, 1979; Marks and Popoff, 1988).

1.1.1.4 Osteoclast Cell Lineage

Granulocyte-macrophage progenitor cells which are found in blood are pluripotent cells capable of maturing into monocytes, macrophages or osteoclasts (Nijweide *et al.*, 1986; Marks and Popoff, 1988; Burger and Nijweide, 1991). These uninucleate precursor cells differentiate to form pre-osteoclasts in blood (Figure 1.1). When pre-osteoclasts enter bone it is believed they are stimulated by osteoblasts and osteocytes to fuse (Aubin *et al.*, 1993). This results in the formation of an osteoclast containing up to 50 nuclei per cell. Osteoclasts are long-lived cells passing through quiescent and active stages in which they degrade calcified bone and cartilage extracellular matrix (Kato and Akisaka, 1994; Zaidi *et al.*, 1993; Howlett, 1980).

1.1.2 Development and Organisation of Bone

Bone development occurs by one of two processes, depending on which type of bone will be formed. Intramembranous ossification is typical of the flat bones which form the skull, while endochondral ossification is the process by which most other bones (including long bones) of the skeleton form (Hall and Newman, 1991). Although the route taken to achieve bone formation is different, the bone that is formed by each process is similar.

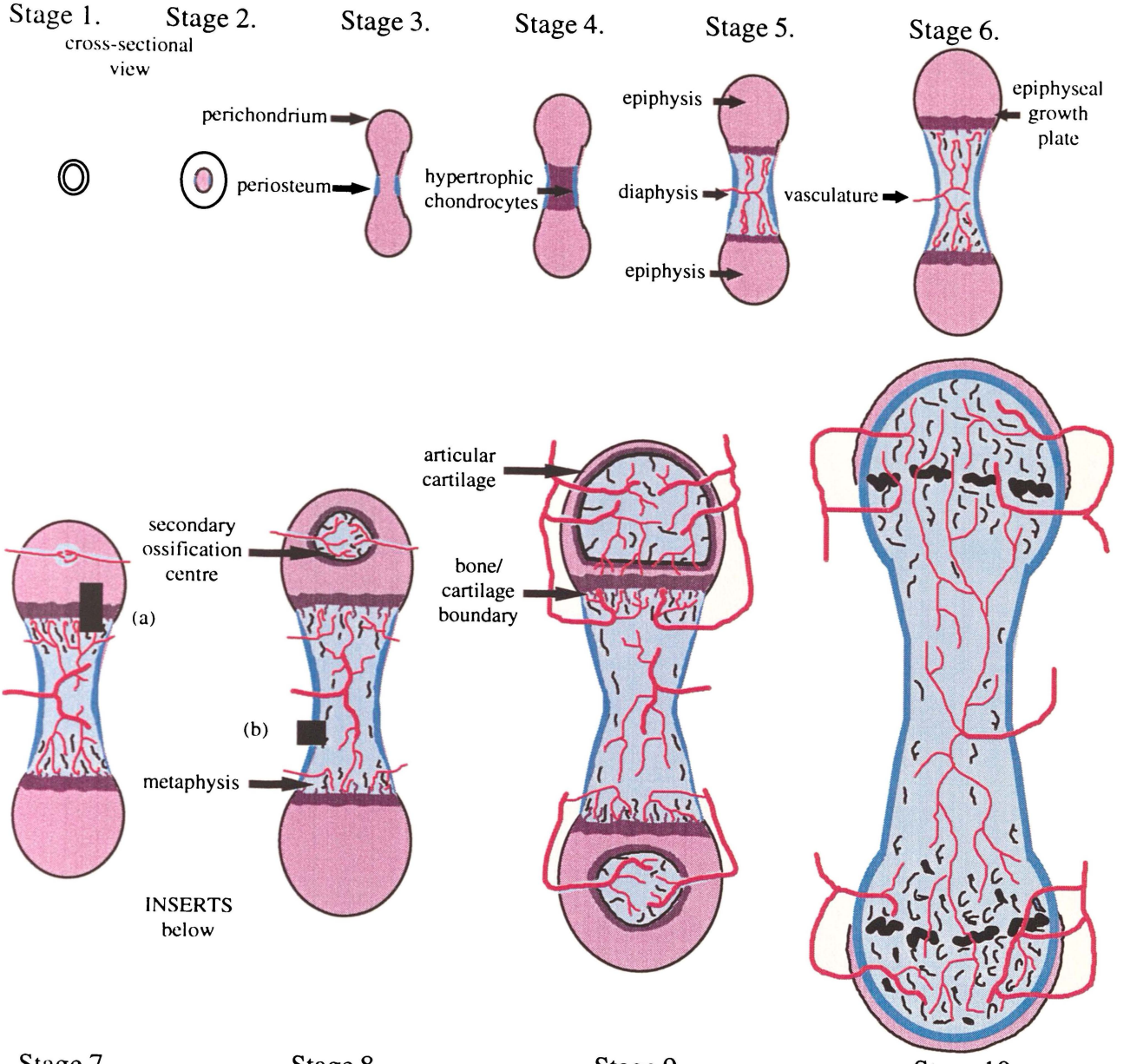
Intramembranous ossification involves formation of bone without a pre-existing cartilage model (Ham and Cormack, 1979). From a layer of loose mesenchyme, a few cells differentiate into osteogenic cells. These subsequently differentiate to form osteoblasts which secrete an extensive extracellular matrix around themselves. The bone matrix absorbs calcium salts, calcifies and osteoblasts further differentiate to osteocytes. This process initiates the differentiation of other mesenchymal cells along this bone cell pathway, resulting in the formation of cancellous bone.

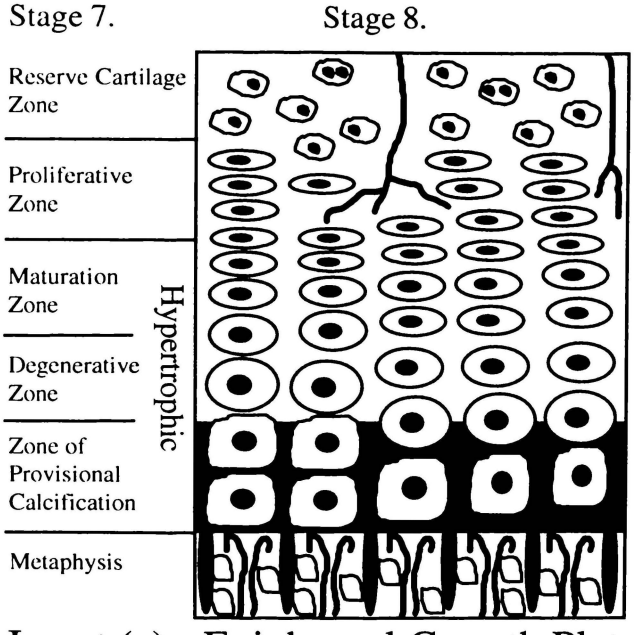
Endochondral ossification is a process where the formation of bone occurs by a more complex route. Most of the skeleton, including the long bones of the limb, require formation of a cartilage template first, which directs positioning of bone formation. Prenatal development of long bones proceeds as shown in Figure 1.2 by the formation of an outgrowth from the trunk (Collin-Osdoby, 1994; Ham and Cormack, 1979; Caplan and Pechak, 1987; Caplan, 1988). The limb bud consists of mesodermal cells surrounded by ectoderm. Mesenchymal cells within the mesoderm of the limb bud condense and differentiate into chondrocytes which grow to produce a cartilage model of future bone. Prior to differentiation, blood vessels egress and cartilage is kept avascular by anti-angiogenic substances (Collin-Osdoby, 1994; Hallmann *et al.*, 1987). The cartilage model increases in size by further differentiation of chondrogenic cells from the perichondrium, chondrocyte cell division and cartilage matrix production. Once the model reaches a critical size, the inner-most chondrocytes form hypertrophic chondrocytes. Chondrocyte hypertrophy coincides with

Figure 1.2
Development of a Typical Human Long Bone
INSERTS: Architecture of Epiphyseal Growth Plate and Periosteum
Formation of bone, growth plate and periosteum shown in longitudinal section.
Areas where cells from the bone and osteoclast cell lineages are located are symbolised in shades of purple.
Areas where cells from the cartilage cell lineage are located are symbolised in shades of blue.
Vascularisation is symbolised in red.

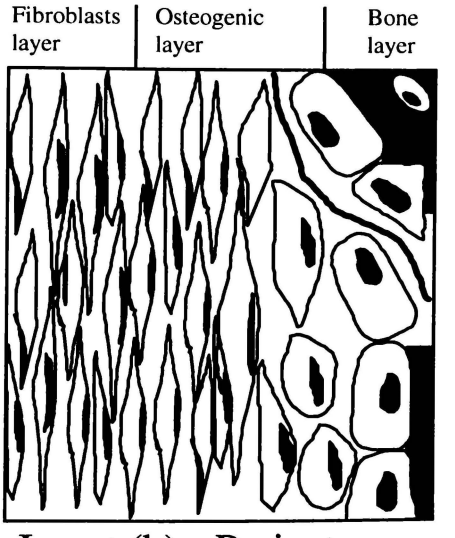
Stage 1. mesodermal limb bud outgrowth (surrounded by ectoderm)
Stage 2. chondrocyte cartilage model (surrounded by perichondrium and periosteum)
Stage 3. cartilage model grows
Stage 4. ossification begins, cartilage cells adjacent to periosteum hypertrophy
Stage 5. vascularisation and bone formation results in a bone bridge (diaphysis)
Stage 6. continued growth in length and width of bone
Stage 7. initiation of secondary centre of ossification in the proximal epiphysis
Stage 8. continued growth of bone and secondary centre of ossification
Stage 9. initiation of secondary centre of ossification in the distal epiphysis
Stage 10. postnatal closure of the epiphyseal growth plate

Source:
Redrawn from information supplied by Bloom and Fawcett (1994) and Iannotti (1990)





Insert (a) - Epiphyseal Growth Plate



Insert (b) - Periosteum

the initiation of primary ossification, as described below. Hypertrophic cells continue to synthesise cartilage matrix, which eventually calcifies when chondrocytes reach maturity.

Throughout this process the cartilage model is surrounded by a layer of perichondrium, which consists of two layers, both from mesenchymal cells. There is an outer layer of connective tissue containing fibroblasts and an inner layer of relatively undifferentiated chondrogenic cells. Chondrogenic cells differentiate into chondrocytes as required, thus adding to growth of the cartilage model. Within the perichondrium at the site where primary ossification will take place, there is a stacked cell layer with osteogenic potential (Caplan, 1988). Osteogenic cells in this layer are derived from mesenchymal cells and have a similar appearance to chondrogenic cells.

Concurrent with changes already described in the interior of the cartilage model, formation of bone at the midsection of this model is initiated by vascularisation of the perichondrium. Blood vessels and associated perivascular cells, which include osteoprogenitor cells, invade the hypertrophying calcified cartilage region. Perivascular cells of the endothelial cell lineage coalesce to form capillaries and establish a suitable environment for the differentiation of osteogenic cells into osteoblasts (Collin-Osdoby, 1994).

Differentiation of the osteogenic cells through a bone cell pathway results in the formation of a primary centre of ossification. This region of perichondrium is now termed the periosteum. By this process, a small collar of bone forms between hypertrophic chondrocytes and the periosteum. The bone matrix calcifies, hypertrophic cells die and they, along with their calcified cartilage matrix, are degraded and absorbed by invading osteoclasts. Bone formation occurs in the spaces vacated by cartilage on remnants of cartilage matrix called trabeculae. This is achieved by the migration of osteogenic cells to this area and their subsequent differentiation. Eventually a bone bridge across the midsection of the cartilage model is formed. The bony midsection continues to grow and form the diaphysis (shaft) of bone. The processes of angiogenesis, bone formation and bone degradation are all essential for the continued replacement of the cartilage model with bone.

The two cartilaginous ends of the original cartilage model, the epiphyses, are now separated by the bony diaphysis. Cartilage at each epiphysis appears as a distinct linear arrangement of chondrocytes at various stages of differentiation which collectively form two separate epiphyseal growth plates at each end of the bone (Figure 1.2).

Based on morphology, the epiphyseal growth plate is divided into four major zones (Robertson, 1990; Brighton, 1984; Howlett, 1979). Chondrocytes maturing at a set location become part of each zone as they proceed through their life cycle. The zone of resting cartilage is located most distal to the diaphysis. In this zone, chondrocytes surrounded by cartilage matrix are considered 'resting' because they do not participate in growth of the growth plate. Chondrocytes in the resting zone become activated to form the zone of proliferating young cartilage. Chondrocytes within this zone proliferate along a perpendicular axis resulting in thin cells stacked in columns. After proliferation, chondrocytes begin to mature and form hypertrophic cells in a zone of maturing cartilage, characterised by cells of various shapes and degrees of maturity. The hypertrophic zone adjacent to the diaphysis is the final stage through which chondrocytes pass. Chondrocytes increase dramatically in size in this zone. The cartilage matrix becomes calcified and hypertrophic cells eventually die and become reabsorbed. The cartilage matrix then provides a scaffolding for the formation of bone in an area called the metaphysis (Figure 1.2).

Later in development, a secondary centre of ossification forms in each epiphysis in a similar way to the process of initial bone formation. The mature epiphysis therefore consists of a thin layer of articular cartilage present on the outside of bone and a narrow disk of cartilage connecting the diaphysis to the secondary ossification centre. This is the epiphyseal growth plate. The region at the bone (diaphysis)-cartilage (growth plate) boundary called the metaphysis, is characterised by cartilage degradation, bone formation and angiogenesis. The periosteum and perichondrium constitute a continuous layer on the outside of mature bone, with potential for bone or cartilage formation depending on vascularisation and whether the layer is adjacent to bone or cartilage.

1.1.3 Blood Supply to Long Bones

Both intramembranous and endochondral ossification occur in association with blood capillaries (Collin-Osdoby, 1994). The vascular system grows and develops to form an extensive network to keep pace with bone growth and development.

1.1.3.1 **Blood Vessel Anatomy**

Blood vessels in a developed bone enter the bone either through the periosteum or perichondrium to maintain independent blood supply to the metaphyses and epiphyses (Figure 1.2) (Skawina *et al.*, 1994; Howlett *et al.*, 1984; Trueta, 1963).

Vessels that enter the epiphysis through the perichondrium form many branches. Some of these vascular canal branches penetrate into the growth plate where they orientate along the plane of growth (Levene, 1964). Vascular canals within the growth plate are spaced at regular distances from each other (Wilsman and Van Sickle, 1971), and although they are found within the proliferative zone, they never extend as far as the hypertrophic zone (Levene, 1964). Cartilage canals consist of an arteriole, a venule, capillaries and loose connective tissue containing fibroblasts and mesenchymal cells (Wilsman and Van Sickle, 1971; Howlett *et al.*, 1984). Pores in the endothelium making up the vasculature allow red blood cells, neutrophils, lymphocytes and macrophages to extravasate into the connective tissue of vascular canals (Wilsman and Van Sickle, 1971). It is believed that cartilage canals assist in nutrition of the epiphysis and that mesenchymal cells located within the canals are involved in interstitial growth of the growth plate (Brookes, 1971).

Blood supply to the diaphysis and metaphysis is carried by vessels found in the periosteum. Branches from this supply extend to the metaphysis but do not enter the growth plate. Vasculature at the boundary between the growth plate and the metaphysis is characterised by capillary loops that lie parallel to the plane of bone growth.

1.1.3.2 **Angiogenesis**

Vasculature is extended in growing bones by the sprouting of new capillaries from a pre-existing network (Coffin *et al.*, 1991). This is the process of angiogenesis. Since capillaries are made up of endothelial cells, angiogenesis is characterised by endothelial cell differentiation, migration and proliferation (Collin-Osdoby, 1994). A major site of angiogenesis in long bones is the metaphysis, adjacent to the degenerating hypertrophic zone of the growth plate. Regularly spaced metaphyseal capillary loops penetrate into the lacunae of degraded hypertrophic columns (Howlett, 1980; Howlett *et al.*, 1984). At the end of each capillary loop saccular sprouts, consisting of endothelial cells, grow into the chondrocytic lacunae, thus extending the vasculature. The endothelium required for this growth is formed by dividing endothelial cells some distance from the advancing capillary tips (Howlett, 1980). Pores in the endothelium allow elements in blood to pass into extravascular tissue (Howlett *et al.*, 1984).

1.1.4 Bone Growth in Length and Width

Longitudinal bone growth is associated with the epiphyseal growth plate and appositional growth in width is associated with the bone surface or periosteum (Kember, 1978; Hert, 1972).

Longitudinal growth of bone occurs through the epiphyseal growth plate via a dynamic process of cartilage formation, degradation and replacement by bone. Cartilage cells divide and differentiate to form hypertrophic cells. An increase in bone width is the net result of relatively less bone resorption and more bone formation on the outer surface of bone. At the cellular level, bone is formed on the outer surface of bone by a process similar to intramembranous ossification described in the previous section. Osteoblasts differentiate from osteogenic cells residing in the periosteum.

1.2 INSULIN-LIKE GROWTH FACTORS IN BONE GROWTH AND DEVELOPMENT

1.2.1 IGF Actions in Bone Growth

It is clear from numerous *in vivo* and *in vitro* studies that IGF-I and IGF-II are important factors involved in the process of bone growth and development described in the previous section. However, it is difficult to determine what are the primary responses and what are the secondary responses to these factors. As yet, no overall unifying model of IGF action in bone has been defined despite the wide range of biological actions reported for IGF-I and IGF-II in this tissue.

From the evidence available, it appears that IGF-I and IGF-II have similar overall actions. They induce cell replication, and stimulate the differentiated functions of target cells, but it must be kept in mind that they do not achieve this alone. Many other growth factors are likely to act synergistically with them to control these processes (Trippel *et al.*, 1993; Hill *et al.*, 1992a; Hill and Logan, 1992b; Hill and De Sousa, 1990a).

1.2.1.1 ***In vivo* Studies**

Evidence has accumulated on the involvement of IGFs in foetal bone growth. An absence of either IGF-I or IGF-II during mouse foetal development results in whole animal growth retardation. At birth, mice homozygous for a targeted disruption of either IGF are 40% smaller than the birth weight of their normal littermates (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993; De Chiara *et al.*, 1990). The phenotype of IGF-I and IGF-II knockout mice are similar. Long bones from null IGF-I and IGF-II mice are described as 1 - 2 days less mature than normal littermates at a given foetal age (Liu *et al.*, 1993). Although detailed information is not available on the histology of bones from these mice, qualitative assessment revealed that in most bones there is a delay in the formation of ossification centres (Liu *et al.*, 1993).

Evidence for IGF-I involvement in postnatal bone growth and development can be found in Laron dwarfs which are a model for postnatal IGF-I deficiency. This human syndrome named Laron syndrome or primary GH (growth hormone) insensitivity is characterised by mutations in the GH receptor gene resulting in people that are shorter and lighter than normal (Laron, 1995). Most Laron dwarfs also have delayed skeletal maturation (Laron and Klinger, 1994; Savage, 1993). Postnatal serum levels of IGF-I in Laron syndrome are negligible (<0.07 U/ml) (Cotterill *et al.*, 1992) The phenotype observed postnatally in Laron syndrome is believed to be largely the result of IGF-I deficiency (Laron, 1995). Evidence to support this comes from studies in which an increase in growth (height and weight) is observed when children with this syndrome are treated systemically with IGF-I (Laron and Klinger, 1994; Heinrichs *et al.*, 1993; Laron *et al.*, 1992; Walker and McNicol, 1992). Although some studies report that GH has additional direct effects on long bone growth (to either stimulate local production of IGF-I or effects independent of IGF-I) it seems that these make only a small contribution to the postnatal phenotype observed in Laron syndrome (Ohlsson *et al.*, 1992b; Baker *et al.*, 1992; Isaksson *et al.*, 1987). Despite the direct effects of GH, Laron dwarfs are a good model for IGF-I deficiency postnatally, but are not a good model in the foetal period.

At birth, Laron dwarfs have a significantly shorter length but normal weight compared to babies without the syndrome (Laron *et al.*, 1966). The growth retardation in Laron dwarfs at birth does not approach the severity (40% reduction) observed in IGF-I deficient mice at birth. This may be because IGF-I production prenatally is controlled independently of GH. When foetal sheep are treated with GH, there are no changes in IGF-I plasma levels but there are modest changes in overall foetal weight (Bauer *et al.*, 1996). It is thought that GH receptors are scarce in the foetus, and that a biological response does not necessarily follow receptor occupancy (Milner and Hill, 1987). The minor intrauterine effects observed in Laron syndrome therefore could be due to an absence of other actions normally carried out by GH in the foetal period.

A number of studies have looked at the effects of over-expression of IGF-I or IGF-II in transgenic mice or rabbits. Results are exclusively for the postnatal period and few analyses have been carried out in bone. There is wide variation in the results obtained from these studies due to the particular promoters used for the transgene, and the level and distribution of transgene expression. Additionally, elevated circulating levels of IGF-I or IGF-II may not have an effect on tissues where normal levels are not rate limiting for a biological response or if these tissues are not responsive to circulating IGF.

Mathews *et al.* (1988) found an increase in weight postnatally with mice transgenic for IGF-I. However no difference between normal and transgenic mice was found in bone length or accumulated bone growth despite a modest increase of 1.5 times the normal circulating level of IGF-I. The local expression of the IGF-I transgene was not investigated in this study (Mathews *et al.*, 1988). In contrast, when IGF-I transgenic mice are crossed with GH-deficient mice, mice carrying both transgenes express IGF-I in the absence of GH and exhibit weight and linear growth indistinguishable from that of their non-transgenic siblings which suggests a role for IGF-I independent of GH (Behringer *et al.*, 1990). It is likely that the difference between these two studies reflects differences in the local production of IGF-I mRNA between these two sets of animals.

In mice containing an IGF-II transgene, there was generally no change observed in overall body weight despite 2 - 3 fold higher serum levels of IGF-II. There was, however, disproportionate growth between some organs postnatally (van Buul-Offers *et al.*, 1995; Wolf *et al.*, 1994; Ward *et al.*, 1994). Bone growth parameters measured by Wolf *et al.* (1994) were not significantly different between transgenic mice and controls.

When administered *in vivo*, either directly into the growth plate or indirectly into an adjacent artery supplying the growth plate, IGF-I has stimulatory effects on bone growth indices such as growth plate width (Guler *et al.*, 1988; Schlechter *et al.*, 1986; Schoenle *et al.*, 1985; Russell and Spencer, 1985); accumulated bone growth (Glassock *et al.*, 1992; Schmid *et al.*, 1989; Guler *et al.*, 1988; Isgaard *et al.*, 1986); protein production (Martínez *et al.*, 1994);

mineral density and bone formation (Ammann *et al.*, 1993; Spencer *et al.*, 1991); longitudinal growth rate (Tobias *et al.*, 1992) and chondrocyte activities at various stages of differentiation (includes cell height and volume and cell cycle time) (Hunziker *et al.*, 1994). These results have all been obtained in postnatal animals against a physiological background of normal or deficient circulating IGF-I levels. Fewer studies investigating the effects of IGF-II administration on long bones have been carried out. Schoenle (1985) found that IGF-II infusion into hypophysectomised rats resulted in an increase in growth plate width. On an equivalent basis, IGF-II was less potent than IGF-I at stimulating this increase (Schoenle *et al.*, 1985). No effect on skeletal growth was found by Glassock *et al.* (1992) when IGF-II was infused for a period of 8 days into neonatal hypophysectomised rats.

Similar studies on the infusion of IGF-I or IGF-II into the foetus have not been reported.

1.2.1.2 *In vitro* Studies

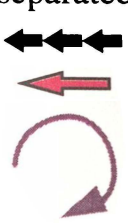
The target cells and biological actions of IGF-I and IGF-II in bone have been better defined by a substantial number of cell culture studies. These include cultures from cartilage, bone and osteoclast cell lineages. Numerous different effects of IGF-I and IGF-II on these cells have been reported. In interpreting these effects it must be kept in mind that cells *in vitro* do no necessarily behave as they do *in vivo* (Erlebacher *et al.*, 1995). Often, isolated cells grown in culture represent a mixed population of cells instead of a single phenotype (Wlodarski, 1990). Also, in an *in vivo* situation, a particular component of the IGF system may not be available to elicit the biological response observed *in vitro*.

Cartilage, bone and osteoclast cells isolated from a range of species (human, rat, mouse, sheep, cow, rabbit and coho salmon), developmental stages (foetal to adult) and stages of differentiation, respond similarly to IGF-I and IGF-II (Figure 1.3). Most studies have focused on the actions of IGF-I, however, both IGFs have been shown to stimulate cell proliferation and processes associated with differentiation (Figure 1.3).

Figure 1.3

In vitro Effects of IGF-I and IGF-II in Bone Cell Lineage's

Effects of IGF-I and IGF-II on cell replication, differentiation, protein and/or RNA synthesis in cell culture or organ culture studies. The effects are separated according to their cell lineage.



Differentiation

Protein/RNA synthesis

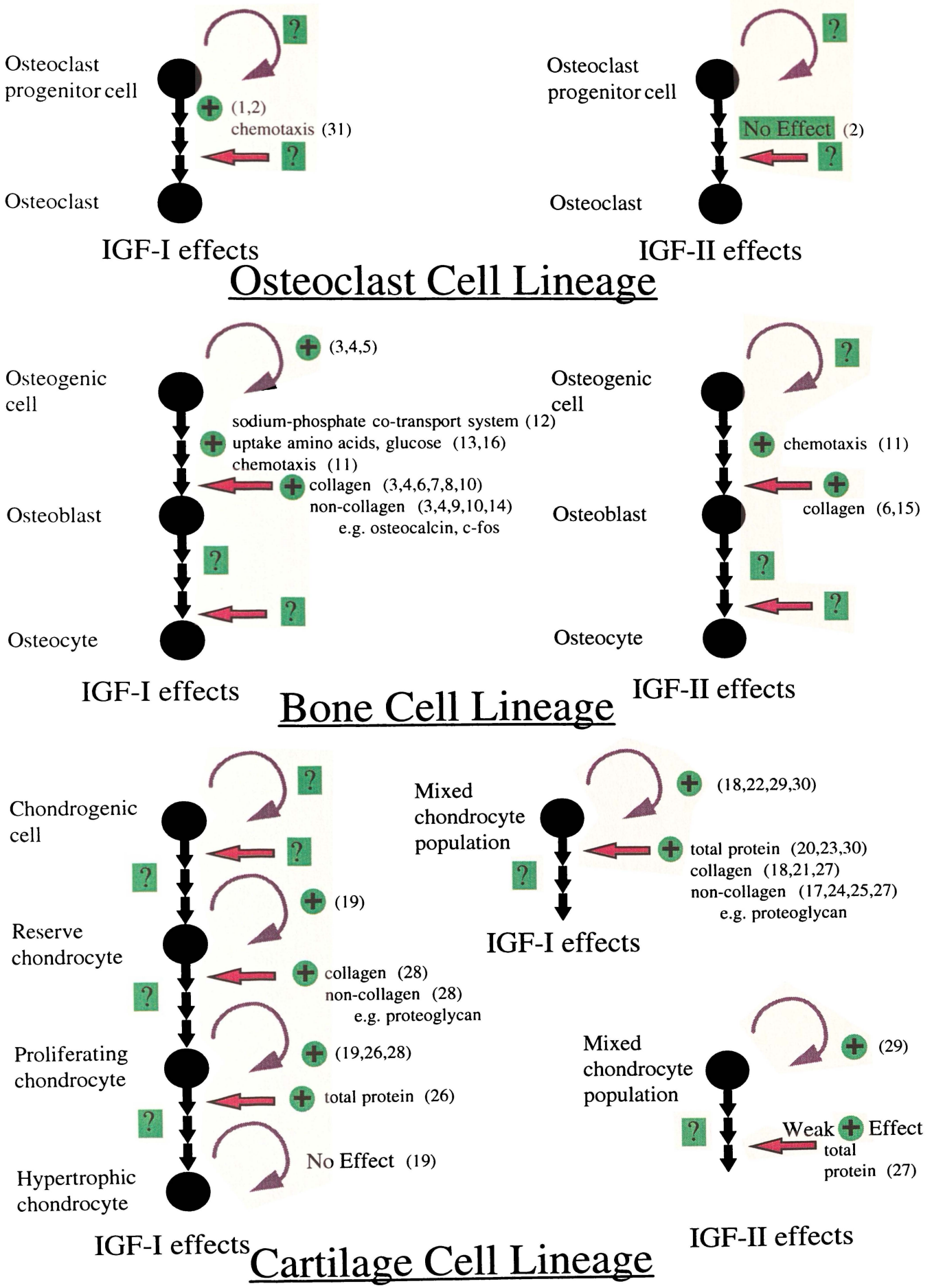
Cell replication

Stimulatory effect

Unknown effect on differentiation

Source:

(1) Slootweg *et al.*, 1992; (2) Mochizuki *et al.*, 1992; (3) Hock *et al.*, 1988; (4) Canalis *et al.*, 1991; (5) Van de Pol *et al.*, 1989; (6) McCarthy *et al.*, 1989a; (7) Raisz *et al.*, 1993; (8) Canalis *et al.*, 1989; (9) Pirskanen *et al.*, 1993; (10) Pfeilschifter *et al.*, 1990; (11) Panagakos, 1993; (12) Campbell *et al.*, 1992; (13) Schmid, 1993; (14) Merriman *et al.*, 1990; (15) Mohan *et al.*, 1992; (16) Schmid *et al.*, 1984; (17) Tyler, 1989; (18) Maor *et al.*, 1993a; (19) Lindahl *et al.*, 1987a; (20) McCormick *et al.*, 1992; (21) Demarquay *et al.*, 1990; (22) Maor *et al.*, 1993b; (23) Salmon and Daughaday, 1957; (24) Sah *et al.*, 1994; (25) Curtis *et al.*, 1992; (26) Hill, 1979; (27) Hill *et al.*, 1992; (28) Böhme *et al.* 1992; (29) Vetter *et al.*, 1986; (30) Ohlsson *et al.*, 1992a; (31) Formigli *et al.*, 1995


Osteoclast progenitor cell
(1,2)
chemotaxis (31)
Osteoclast
IGF-I effects
Osteoclast progenitor cell
No Effect (2)
Osteoclast
IGF-II effects
Osteoclast Cell Lineage
Osteogenic cell
(3,4,5)
sodium-phosphate co-transport system (12)
uptake amino acids, glucose (13,16)
chemotaxis (11)
collagen (3,4,6,7,8,10)
non-collagen (3,4,9,10,14)
e.g. osteocalcin, c-fos
Osteoblast
Osteocyte
IGF-I effects
Osteogenic cell
chemotaxis (11)
collagen (6,15)
Osteoblast
Osteocyte
IGF-II effects
Bone Cell Lineage
Chondrogenic cell
Reserve chondrocyte
(19)
collagen (28)
non-collagen (28)
e.g. proteoglycan
Proliferating chondrocyte
(19,26,28)
total protein (26)
Hypertrophic chondrocyte
No Effect (19)
IGF-I effects
Mixed chondrocyte population
(18,22,29,30)
total protein (20,23,30)
collagen (18,21,27)
non-collagen (17,24,25,27)
e.g. proteoglycan
IGF-I effects
Mixed chondrocyte population
(29)
Weak Effect
total protein (27)
IGF-II effects
Cartilage Cell Lineage

As part of their role in the stimulation of differentiation, IGFs increase levels of total protein (Figure 1.3). This is achieved by an increase in protein production, measured at the level of RNA or protein, or through the prevention of protein degradation (Pirskanen *et al.*, 1993; Canalis *et al.*, 1991; Tyler, 1989; McCarthy *et al.*, 1989a). Both collagen and non-collagen proteins (proteoglycan, osteocalcin and c-fos) increase when IGFs are added in culture (Figure 1.3). Consistent with these actions, IGF-I has been shown to increase the uptake of amino acids and glucose in osteoblast cell cultures (Schmid, 1993; Schmid *et al.*, 1984).

Additional actions of IGF-I and IGF-II have been reported in osteoblast cells. IGF-I was shown to stimulate the transport of inorganic phosphate from the extracellular to the intracellular compartment by means of a sodium dependent transport system (Campbell *et al.*, 1992). Trancellular transport of inorganic phosphate to the extracellular matrix may be important for matrix calcification. IGF-I and IGF-II also stimulate chemotaxis, a process that enables osteoblasts to be recruited to the appropriate site for bone formation (Panagakos, 1993).

When compared directly, IGF-I is 4 to 7 times more potent than IGF-II at stimulating DNA, collagen and non-collagen synthesis in rat calvariae culture (McCarthy *et al.*, 1989b; Luyten *et al.*, 1988). Other studies found that potency depended on the developmental age the cells were isolated from (Trippel *et al.*, 1993; Vetter *et al.*, 1986). IGF-II was more effective than IGF-I at stimulating clonal growth of foetal chondrocytes whereas IGF-I was more effective in adult chondrocytes (Vetter *et al.*, 1986). Relative potencies of IGF-I and IGF-II may be due to their relative affinities for the Type I IGF receptor (Section 1.2.2), culture conditions and the role of the Type II IGF receptor at preventing actions of IGF-II through the Type I IGF receptor.

1.2.1.3 **Role of IGF-I and IGF-II in Angiogenesis**

It has been postulated that the coupling of chondrogenesis (cartilage formation) and ossification (bone formation) during endochondral ossification may be partly controlled by the presence of a vascular system in the metaphysis (Baron *et al.*, 1994; Caplan and Pechak,

1987). Continued growth of the vascular system to keep pace with bone growth is therefore an important process. The steps involved in angiogenesis include degradation of the vascular basement membrane and interstitial matrix by endothelial cells, migration of endothelial cells, proliferation of endothelial cells and formation of capillary loops (Sato *et al.*, 1993).

IGFs have been shown to stimulate endothelial cells which are important for these steps in angiogenesis (Nicosia *et al.*, 1994; Grant *et al.*, 1993). IGF-I and IGF-II are mitogenic for endothelial cells (Sato *et al.*, 1993; Streeten and Brandi, 1990). They stimulate functions in cultured microvessel endothelial cells including an increase in the production of proteoglycans (Bar *et al.*, 1988; Bar *et al.*, 1984) IGF-I has additionally been shown to induce chemotactic responses associated with endothelial cell migration and stimulate tube formation in bone endothelium indicating an important role in directing angiogenesis (Fiorelli *et al.*, 1994; Nakao-Hayashi *et al.*, 1992).

1.2.2 IGF Signal Transduction

In order for IGFs to achieve biological actions, IGF receptors must be present. IGF-I and IGF-II bind to the insulin, Type I and Type II IGF receptors with differing affinities (*Type I IGF receptor* binding affinity: IGF-I > IGF-II >> insulin; *Type II IGF receptor* binding affinity: IGF-II >> IGF-I, does not bind insulin; *insulin receptor* binding affinity: insulin >>> IGF-I, does not bind IGF-II) (Massagué and Czech, 1982; Chernausek *et al.*, 1981; Kasuga *et al.*, 1981). There is also some evidence that IGFs bind with low affinity to hybrid IGF/insulin receptors (Jones and Clemmons, 1995).

The biological actions of IGF-I and IGF-II are largely the result of their binding to the Type I and Type II IGF receptors (LeRoith *et al.*, 1995; Jones and Clemmons, 1995; Lau *et al.*, 1994; Liu *et al.*, 1993). There is no evidence to support *in vivo* IGF actions through the insulin or IGF-I/insulin receptors (Jones and Clemmons, 1995).

Most of the reported cellular effects of the IGFs are believed to be mediated by binding of both peptides to the Type I IGF receptor (Figures 1.4 and 1.5). IGF-II binding to the Type II IGF receptor is believed to modulate the availability of IGF-II to the Type I IGF receptor rather than a signalling event and direct cellular effects through this receptor. The Type II IGF receptor therefore probably functions primarily as a degradative pathway to remove IGF-II from the extracellular environment (Liu *et al.*, 1993; Lau *et al.*, 1994; Jones and Clemmons, 1995; Schlessinger *et al.*, 1995). The Type II IGF receptor is structurally different from the insulin and Type I IGF receptor and has a dual role as the cation independent mannose-6-phosphate receptor (Morgan *et al.*, 1987; MacDonald *et al.*, 1988; Kiess *et al.*, 1988). Although a few reports suggest that IGF-II mitogenic activity occurs through the Type II IGF receptor (Okamoto *et al.*, 1990; Mathieu *et al.*, 1990; Tally *et al.*, 1987; Blanchard *et al.*, 1988), other studies found that the mitogenic effect of IGF-II was not affected when the Type II IGF receptor was blocked with specific antibodies (Hartmann *et al.*, 1992; Adashi *et al.*, 1990; Mottola and Czech, 1984; Kiess *et al.*, 1987; Conover *et al.*, 1986). Blocking the Type I IGF receptor, however, was able to impair IGF-II function (Furlanetto *et al*, 1987; Conover *et al.*, 1986). In addition to the involvement of the Type I and Type II IGF receptors in the biological actions of IGF-II, Baker *et al.* (1993) report evidence of IGF-II action through an additional unknown receptor.

Consistent with the reported actions of IGF-I and IGF-II described in the previous section, IGF receptors are found in foetal and postnatal chondrocyte and osteoblast cells cultured from rat, human and ruminant species (Figure 1.4). IGF-I and IGF-II binding sites, determined by ligand binding studies, or Type I and Type II IGF receptors detected at the level of protein (using specific antibodies) or RNA, are widely and similarly distributed (Figure 1.4).

Type I and Type II IGF receptors are present on chondrocytes throughout the growth plate (Figure 1.4). Highest abundance of IGF-I binding sites has been found in the proliferative zone (Makower *et al.*, 1989). It is in this zone that most studies have reported actions for IGF-I (Figure 1.3). Several reports have shown the presence of Type I and Type II IGF receptors on osteoblasts, however, no studies have determined whether there are receptors on

Figure 1.4

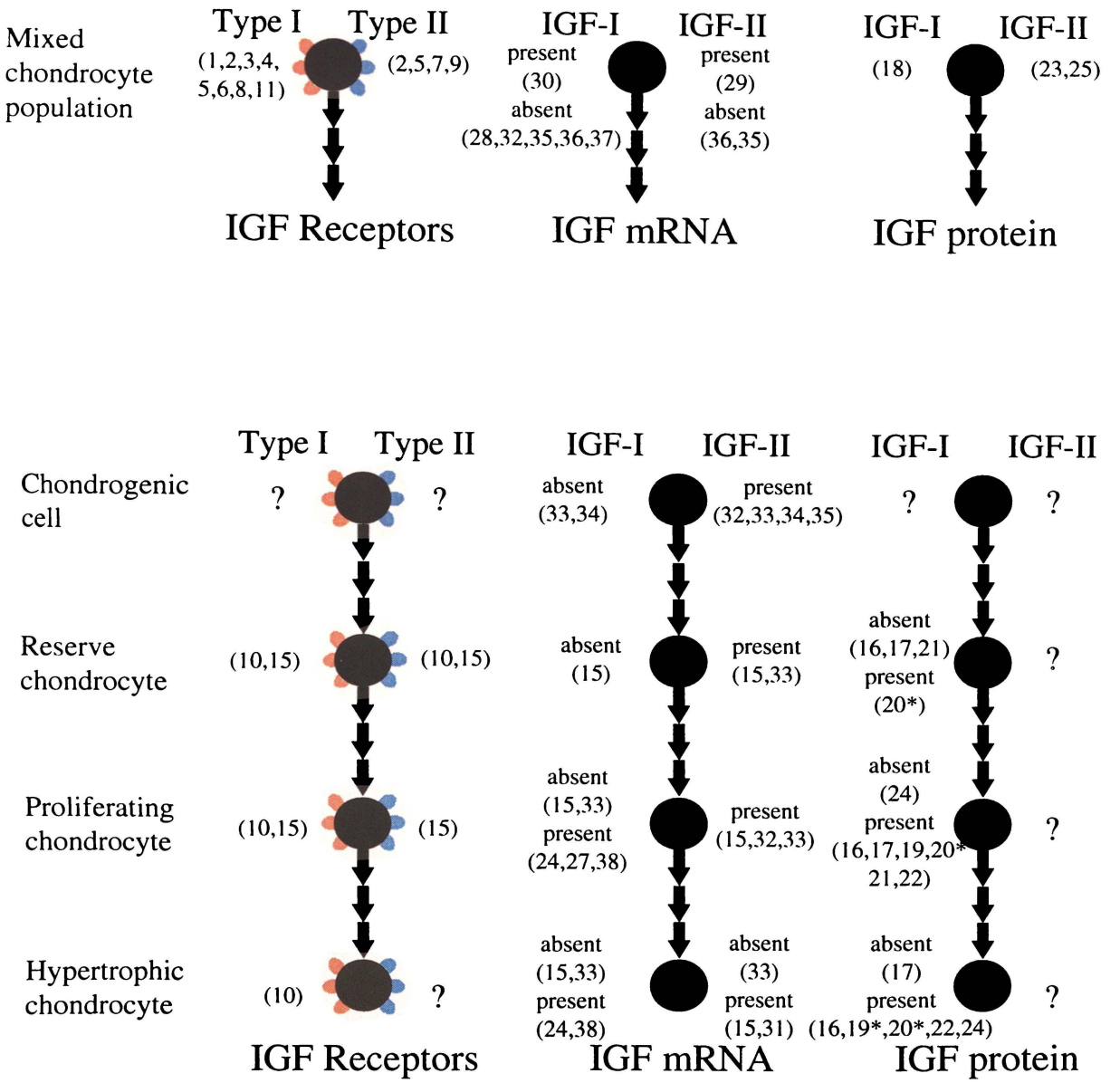
Summary of the Identification of IGF-I and IGF-II Binding Sites (receptors), Protein and mRNA in Bone Cell Lineage's

- IGF-I binding site
- IGF-II binding site
- * Antibody cannot distinguish between IGF-I and IGF-II

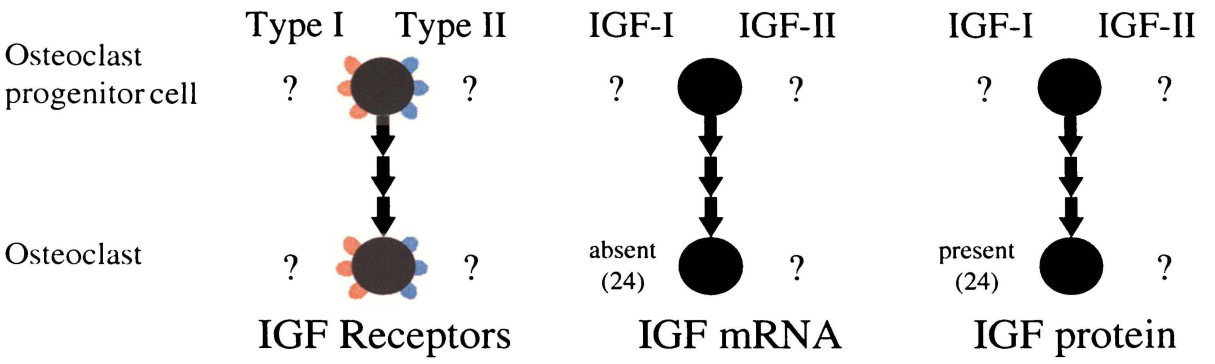
Mixed Chondrocyte Population includes chondrocytes from the growth plate, articular surface, and rib cartilage.

Source:

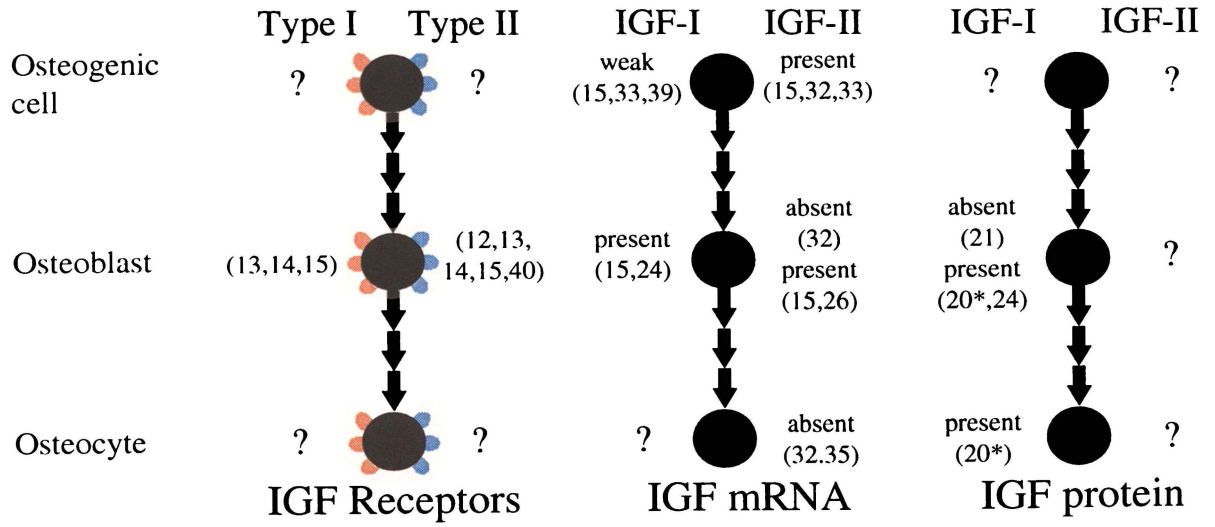
(1) Doré *et al.*, 1994; (2) Trippel *et al.*, 1988; (3) Verschure *et al.*, 1994; (4) Watanabe *et al.*, 1985; (5) Jansen *et al.*, 1989; (6) Schalch *et al.*, 1986; (7) Ashton and Soul, 1984; (8) Trippel *et al.*, 1983; (9) Sessions *et al.*, 1987; (10) Makower *et al.*, 1989; (11) Bentham, 1993; (12) Mohan *et al.*, 1989b; (13) Centrella, 1990; (14) Slootweg *et al.*, 1990; (15) Wang *et al.*, 1995; (16) Simon and Cooke, 1988; (17) Nilsson, 1986; (18) Schneiderman *et al.*, 1995a; (19) Hill *et al.*, 1989; (20) Ralphs and Hill, 1990; (21) Hansson *et al.*, 1988; (22) Leach and Twal, 1994; (23) Lee *et al.*, 1990; (24) Lazowski *et al.*, 1994; (25) Hill and De Sousa, 1990; (26) McCarthy *et al.*, 1992; (27) Nilsson *et al.*, 1990; (28) Middleton *et al.*, 1996; (29) Stylianopoulou *et al.*, 1988; (30) Isgaard *et al.*, 1988a; (31) Hill *et al.*, 1994; (32) Beck *et al.*, 1988; (33) Shinar *et al.*, 1993; (34) Streck *et al.*, 1992; (35) Beck *et al.*, 1987; (36) Han *et al.*, 1987; (37) Middleton and Taylor, 1992; (38) Hanna *et al.*, 1995; (39) Birnbaum *et al.*, 1993; (40) Martinez *et al.*, 1995



Cartilage Cell Lineage



Osteoclast Cell Lineage



Bone Cell Lineage

cells at other stages of differentiation (Figure 1.4) In a model of embryonic bone formation, Type II IGF receptors were found to be highest during developmental stages when cells of the bone cell lineage were most active (Yu *et al.*, 1993). Although IGF-I has been reported to have some actions in the osteoclast cell lineage, no studies have reported whether IGF receptors are present in cells from this lineage (Figure 1.4) (Slootweg *et al.*, 1992; Mochizuki *et al.*, 1992).

The presence of Type I IGF receptors has been demonstrated on endothelial cells and arteries (Fiorelli *et al.*, 1994; Sidaway *et al.*, 1990). This is consistent with a proposed role for IGF-I and IGF-II in angiogenesis at the cartilage-bone boundary (Baron *et al.*, 1994; Caplan and Pechak, 1987).

1.2.3 IGF Distribution

The location of IGF receptors in bone strongly implies a role for IGFs. However, any biological response requires the presence of IGF-I and/or IGF-II.

Many studies have detected IGF-I and IGF-II in whole bone extracts and localised IGFs to regions within bone from several species (human, chicken, rat, mouse and pig) (Figure 1.4) (Leach and Twal, 1994; Frolik *et al.*, 1988; Mohan *et al.*, 1988a). Approximately nine times higher levels of IGF-II compared to IGF-I are present in bone derived from the adult human hip (Frolik *et al.*, 1988; Mohan *et al.*, 1988a).

IGF-I has been found in cartilage, bone and osteoclast cell lineages (Figure 1.4), however not all studies agree on the presence or absence of IGF-I within some cartilage zones of the growth plate (Figure 1.4). Although there is general agreement on the presence of IGF-I in proliferative chondrocytes, the presence of IGF-I in the reserve and hypertrophic zones is not certain (Figure 1.4).

Few studies have described the cellular localisation of IGF-II in bone. Although some studies have demonstrated the release of IGF-II from foetal chondrocytes (Hill and De Sousa, 1990a; Lee *et al.*, 1990), the localisation of IGF-II within the bone and osteoclast cell lineages has not been described.

1.2.4 Source of IGF-I and IGF-II

The source of IGF-I and IGF-II can be by local production or from the circulation (Figure 1.5). Although the source of circulating IGF has yet to be completely determined, IGF-I and IGF-II are probably derived from many tissues and organs since there is widespread expression of IGF in the body (Listrat *et al.*, 1994; Boulle *et al.*, 1993; Delhanty and Han, 1993; Pell *et al.*, 1993; Dickson *et al.*, 1990; O'Mahoney *et al.*, 1991). For the purposes of this thesis circulating IGF will be referred to as an endocrine source of IGF capable of endocrine actions in target tissues. However, it is acknowledged that circulating IGFs are likely to be derived from endocrine glands and non-endocrine tissues, and are therefore not strictly endocrine hormones.

The relative contributions of locally produced versus endocrine derived IGF-I and IGF-II have been debated with studies on local production of IGF-I in bone showing considerable variation between and within species and throughout development. It is likely that *in vivo* both endocrine and paracrine/autocrine models of IGF action operate to supply bone, osteoclast and cartilage cell lineages with appropriate levels of IGF-I and IGF-II.

1.2.4.1 **Local IGF-I and IGF-II Production**

Widespread expression of IGFs and the Type I receptor in bone makes a strong case for the concept that actions of IGF are exerted locally, either on their cells of synthesis (an autocrine mode of action), or on nearby cells (a paracrine mode of action) (D'Ercole, 1991).

1.2.4.1.1 *Cell Culture Studies*

Some studies have investigated *in vitro* production of IGF-I and IGF-II in isolated chondrocyte and bone cells. IGF-I was barely detectable in conditioned media derived from

Figure 1.5

Summary of Proposed Pathways of IGF-I and IGF-II Action

Model of IGF action. Proposed pathways that lead to alterations in the expression of various IGF responsive genes. Activation of these pathways results in longer-term effects observed with IGF, including growth and differentiation

IGF source (circulating, local)

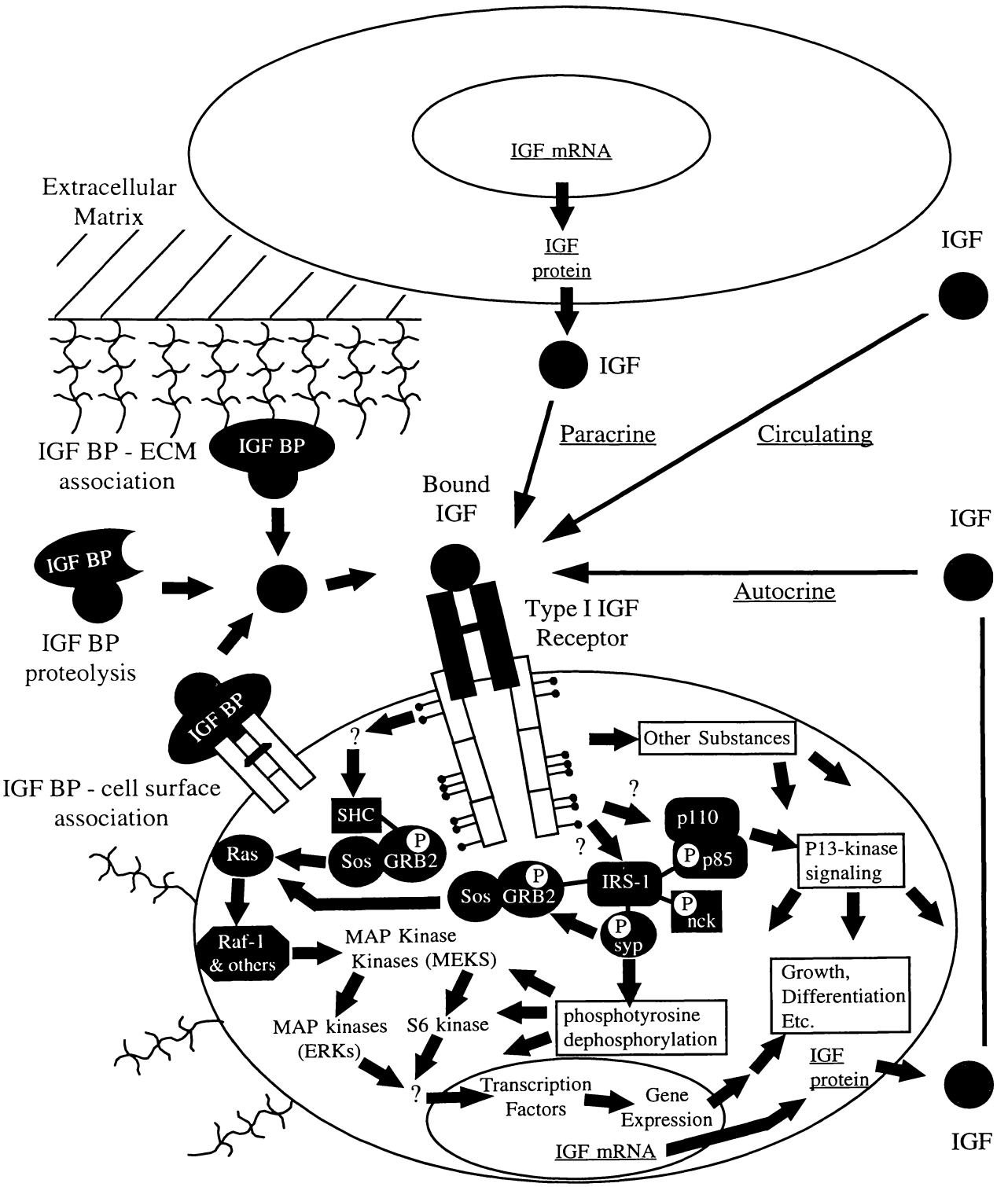
Transport and presentation to IGF Type I receptor (IGF BP-extracellular matrix association, IGF BP proteolysis, IGF BP-cell surface association)

Cell binding

Intracellular signaling pathways through the Type I IGF receptor

Source:

Redrawn from Jones and Clemmons, 1995; LeRoith *et al.*, 1995


IGF mRNA
Extracellular Matrix
IGF protein
IGF
IGF
Paracrine
Circulating
IGF BP - ECM association
IGF BP
Bound IGF
IGF
IGF BP
Autocrine
IGF BP proteolysis
Type I IGF Receptor
IGF BP
IGF BP - cell surface association
?
Other Substances
SHC
?
p110
P
Sos
GRB2
P
Ras
?
P
p85
P13-kinase signaling
P
Sos
GRB2
IRS-1
P
nck
P
syp
Raf-1 & others
MAP Kinase Kinases (MEKS)
Growth, Differentiation Etc.
MAP kinases (ERKs)
S6 kinase
phosphotyrosine dephosphorylation
IGF protein
?
Transcription Factors
Gene Expression
IGF mRNA
IGF

the postnatal rabbit, embryonic mouse and foetal lamb (Bhaumick, 1993; Hill and De Sousa, 1990a; Froger-Gaillard *et al.*, 1989). In contrast, significant levels of IGF-II were detected in conditioned media from the same chondrocytes (Bhaumick, 1993; Hill and De Sousa, 1990a; Froger-Gaillard *et al.*, 1989). These results suggest IGF-II but not IGF-I is synthesised locally in cartilage cells. In a bone cell culture enriched for osteoblasts, IGF-I and IGF-II were both measured in conditioned media (Mohan *et al.*, 1990; Canalis *et al.*, 1988; Mohan *et al.*, 1988b).

1.2.4.1.2 *IGF-I and IGF-II mRNA Synthesis*

Studies in human, rat and mouse have found either an absence or very low levels of IGF-I mRNA in foetal and neonatal cartilage cells (Figure 1.4) (Wang *et al.*, 1995; Shinar *et al.*, 1993; Streck *et al.*, 1992; Nilsson *et al.*, 1990; Beck *et al.*, 1988; Beck *et al.*, 1987; Han *et al.*, 1987). Other studies report the presence of IGF-I mRNA in adult rat cartilage cells (Figure 1.4) (Hanna *et al.*, 1995; Lazowski *et al.*, 1994; Nilsson *et al.*, 1990; Isgaard *et al.*, 1988a). It is possible that upregulation of IGF-I mRNA levels occurs in the postnatal period as has been shown to occur in other tissues during development (Burnside and Cogburn, 1992; Hoyt *et al.*, 1988).

Although IGF-I mRNA does not appear to be present during foetal development, high levels of IGF-II mRNA have consistently been found in cartilage cells throughout prenatal bone development (Wang *et al.*, 1995; Hill *et al.*, 1994; Streck *et al.*, 1992; Stylianopoulou *et al.*, 1988). These results suggest IGF-II and not IGF-I are produced locally in foetal growth plates.

Fewer studies have focused attention on the presence of IGF mRNA in cells from the osteoblast or osteoclast cell lineages. In those studies where it has been described, IGF-I and IGF-II mRNA are localised to osteoblasts and the periosteum, but not osteocytes (Wang *et al.*, 1995; Lazowski *et al.*, 1994; Birnbaum and Bowsher, 1993; Shinar *et al.*, 1993; McCarthy *et al.*, 1992; Beck *et al.*, 1988; Beck *et al.*, 1987). It is unclear whether cells of the osteoclast cell lineage express IGF-I or IGF-II, since only one study showing an absence of IGF-I mRNA in osteoclasts has been reported (Lazowski *et al.*, 1994).

1.2.4.2 **Endocrine IGF-I and IGF-II**

The presence of IGF-I and IGF-II in bone may be the result of production in other tissues and transport to bone via the circulation. Indirect evidence for this comes from the fact that the level of circulating IGFs is high both prenatally and postnatally in various species including sheep (Roberts *et al.*, 1990; Mesiano *et al.*, 1989; Gluckman and Butler, 1985; Gluckman and Butler, 1983; Van Vliet *et al.*, 1983). Furthermore, IGF-I has been shown to correlate with foetal weight (Carr *et al.*, 1995) and administration of exogenous IGFs into the circulating system can cause growth promoting actions in bone (see section 1.2.1.1).

Circulating IGFs are readily available to all parts of bone, not only because of the extensive capillary network, but also because the endothelium of metaphyseal and growth plate capillaries are fenestrated and frequently discontinuous, thus allowing blood elements to easily traverse the capillary endothelium (Howlett, 1980). However, it is unclear whether IGF-I or IGF-II are able to penetrate through cartilage matrix in the growth plate to exert their biological effects, given evidence that higher molecular weight solutes are excluded from entering the matrix in articular cartilage (Maroudas, 1976).

One recent report showed that endogenously added IGF-I, but not IGF-I BP complexes, is able to penetrate into cartilage matrix (Schneiderman *et al.*, 1995a). It was calculated that free IGF-I available *in vivo* from synovial fluid and serum, although consistent with levels of IGF-I extracted from articular cartilage, could not account for the much higher levels of IGF-I required to act in chondrocyte cell culture (Schneiderman *et al.*, 1995a; Schneiderman *et al.*, 1995b). Schneiderman *et al.* (1995a) therefore propose that IGF-I is unable to have a biological effect in articular cartilage. Unlike chondrocytes, access to target bone cells does not require transport through extracellular matrix since osteoblasts secrete their matrix in a single direction which is away from the vasculature (Caplan and Pechak, 1987).

1.2.5 IGF Binding proteins - Transporters and Modulators of IGF Action

Whether IGF-I and IGF-II are from an endocrine, paracrine or autocrine source, they are found in tissue and plasma almost exclusively bound to specific binding proteins. Only a small portion of unbound IGF is detectable (Drop *et al.*, 1992). Association of IGFs with particular binding proteins (IGF BPs) is believed to be important for transporting IGF in plasma, for regulating the efflux of IGFs from the vascular space, for prolonging the half-life of IGFs, for maintaining IGFs in an inert state in the circulation and the extracellular matrix, and for delivering IGFs to IGF receptors for their biological action (Jones and Clemmons, 1995).

There is considerable variation in the literature regarding the level and characterisation of IGF binding proteins in bone. Depending on the cell line, different patterns of IGF BP production are found (Hassager *et al.*, 1992). This variation is largely due to few studies being able to fully characterise the identity of each IGF BP. Characterisation is usually restricted to size and the ability to bind to IGF on a Western Ligand Blot. However, of the six known IGF BPs, five are believed to be either produced by cells in bone or have been located at particular sites within bone.

In rabbit, bovine and ovine cartilage, IGF BPs of 20, 22, 24, 29, 30, 33, 34, 39 and 43 kDa have been reported (Sunic *et al.*, 1995; Olney *et al.*, 1993; Froger-Gaillard *et al.*, 1989). The multiple sizes reported for particular IGF BPs can be due to different glycosylation forms or products of degradation (Drop *et al.*, 1992). The 33 kDa species is believed to be IGF BP-2, while the 39 and 43 kDa species are different glycosylation forms of IGF BP-3 (Olney *et al.*, 1993). IGF BP-5 corresponds to the 29 - 30 kDa species (Sunic *et al.*, 1995). While it is found in *in vitro* studies that these IGF BPs are expressed in cells derived from the growth plate, barely detectable levels of IGF BP-5 and -6 mRNA have only been identified in the reserve cartilage zone of mice growth plates by *in situ* hybridisation (Wang *et al.*, 1995).

Bone cell cultures abundantly produce IGF BP-3, -4 and -5 while osteoblasts *in situ* express IGF BP-2, -3, -4, -5 and -6 mRNA (Wang *et al.*, 1995; Okazaki *et al.*, 1994; Durham *et al.*, 1994; Mohan *et al.*, 1992; Hassager *et al.*, 1992; Mohan *et al.*, 1990; Scharla *et al.*, 1991; Tørring *et al.*, 1991; Mohan *et al.*, 1989a). Variation in expression and production of IGF BP mRNA and protein was found in a developmental model of the rat osteoblast cell lineage. Maximal levels of IGF BP-2 and IGF BP-5 mRNA were present in proliferating pre-osteoblasts, while mature osteoblasts showed maximal expression of IGF BP-3, -4 and -6 (Birnbaum and Wiren, 1994).

It is important to define which IGF BPs are present and where, for each IGF BP has unique characteristics, affinity for IGFs and activities. At the tissue level, factors such as cell surface association, extracellular matrix association, phosphorylation and proteolysis modulate IGF BP affinity for IGFs and thereby IGF activity (Figure 1.5) (Jones and Clemmons, 1995).

1.3 AIMS OF THIS THESIS

The aim of the present study was to identify the potential role IGFs play in the growth and development of foetal sheep long bones, by determining which cells during bone development were likely to respond to IGF-I or IGF-II *in vivo*. To achieve this, components of the IGF system (IGF-I and IGF-II mRNA, IGF-I and IGF-II peptides, Type I and Type II IGF receptors, IGF BP proteins and IGF BP mRNA) were localised in long bones from various ages during foetal development. The availability and localisation of blood-derived IGF-I in bone was determined in animals following the infusion of labelled IGF-I into foetal animals *in utero*.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes the procedures and methodology used for the studies described in chapters 4, 5 and 6, along with the source of materials and the composition of solutions.

2.1 MATERIALS

2.1.1 Source of Materials Used

2.1.1.1 **Probe Templates**

Templates for riboprobe synthesis were subcloned as required into pGEM 3 or its relative pGEM 3Zf+ to allow efficient *in vitro* transcription for *in situ* hybridisation. Templates were designed to be within the range 100 - 700 bp (with the exception of rRNA). Problem sequences within the multiple cloning site of parent (e.g. pbluescript SK+) or target (pGEM 3) plasmids were excluded (Witkiewicz *et al.*, 1993). Templates for riboprobe or DNA probe synthesis were chosen to minimise regions of homology within a gene family. For example, regions of IGF-I were chosen that had lowest homology to sheep IGF-II mRNA. Where small amounts of homology existed, a higher hybridisation stringency was used.

2.1.1.1.1 *IGF-I*

Human IGF-I cDNA template was obtained from M. Jansen. It consisted of a 203 bp *Sau* 3A/*Sau* 3A insert in pUC 13 (Jansen *et al.*, 1983). The cDNA encodes part of the A domain of mature IGF-I, the E domain found in unprocessed IGF-I and a small stretch of 3´

untranslated region. The 203 bp cDNA sequence was subcloned into pGEM 3 by A. Molenaar and used for riboprobe and DNA probe synthesis.

Ovine IGF-I cDNA template was obtained from E. Wong. It consisted of a 790 bp fragment (A21) contained in the plasmid pTZ18R-B (Wong *et al*, 1989). The cDNA was restricted to produce two different DNA probes for Northern analysis. The first probe was a 264 bp *Bam* HI/*Xho* I fragment representing the C and A domains of mature IGF-I, the E domain of unprocessed IGF-I and a small stretch of 3´ untranslated sequence. The second probe was a 203 bp *Sau* 3A/*Sau* 3A fragment equivalent to the human IGF-I cDNA described above.

2.1.1.1.2 *IGF-II*

Human IGF-II cDNA was obtained from G. Bell. It consisted of a 1 095 bp fragment with *Pst* I end linkers in pKT218 (Bell *et al.*, 1984). A 561 bp fragment was subcloned by R. Wilkins into pGEM 3. The 552 bp template contains the protein coding region of mature IGF-II, the unprocessed regions 5' and 3' (E domain) of pre-pro-IGF-II, and a small stretch of 3' untranslated sequence. This template was used for riboprobe and DNA probe synthesis.

Ovine IGF-II cDNA template was obtained from E. Wong (Ohlsen *et al.*, 1994). It consisted of a 1225 bp fragment. The cDNA was restricted to produce two non-overlapping DNA probes for Northern analysis. The first probe was a 388 bp *Bg*l I/*Sty* I fragment encoding a region within that encompassed by the hIGF-II probe described above. The second probe was a 350 bp *Sty* I/*Dra* I fragment 3' to the first probe. It coded for a portion of the E domain of unprocessed IGF-II and 3' untranslated sequence.

2.1.1.1.3 *IGF binding proteins -2, -4 and -5*

Rat IGF binding protein cDNA templates were obtained from S. Shimasaki (Shimasaki *et al.*, 1991a). They were all contained in pbluescript SK+ with sizes of 397 bp (IGF BP-2), 444 bp (IGF BP-4) and 300 bp (IGF BP-5). All cDNAs encoded a region within their appropriate mature binding protein. Each fragment was subcloned into pGEM 3 for riboprobe and DNA probe synthesis.

Ovine IGF binding proteins -2 and -5 were obtained from V. Han (Delhanty and Han, 1993). The cDNAs contained in pbluescript KS+ were restricted to produce a 690 bp BP-2 probe and a 444 bp BP-5 probe for Northern analysis and *in situ* hybridisation. Both cDNAs encoded a region within their appropriate mature binding protein.

2.1.1.1.4 *ribosomal RNA*

A human ribosomal RNA cDNA template was obtained from R. Schmickel (Erickson *et al.*, 1981). It consisted of a 7.3 kb fragment contained in pBR322. A 1 600 bp portion was subcloned by A. Molenaar into pGEM3. This region corresponds to part of the 28S ribosomal RNA coding region. This template was used for riboprobe and DNA probe synthesis

2.1.1.1.5 *α skeletal actin*

Bovine α skeletal actin cDNA template was obtained from H. Davey (Davey and Wildeman, 1995). It consisted of a 233 bp fragment in pTZ18 coding for the 3' untranslated region of α skeletal actin. This template was used for synthesis of an antisense riboprobe and DNA probe.

2.1.1.1.6 *α lactalbumin*

Bovine α lactalbumin cDNA was obtained from W. Hurley (Hurley and Schuler, 1987). It consisted of a 703 bp insert subcloned by P. L'Hullier into pRc/CMV (sense with respect to the T7 promoter) and pTF29+ (antisense with respect to the T7 promoter). These templates were used for riboprobe synthesis.

2.1.1.1.7 *poly* A^+

A template for production of a poly A riboprobe (sense) and poly T riboprobe (antisense) was constructed by A. Molenaar (Molenaar, 1995). The 78 bp template was inserted into pGEM 3.

2.1.1.2 **Antibodies**

All antibodies have been developed for use in sheep.

2.1.1.2.1 *IGF-I*

Anti-human IGF-I polyclonal antisera (rabbit) was obtained from GroPep Pty. Ltd, Australia.

2.1.1.2.2 *IGF-II*

Monoclonal antibody against rat IGF-II derived from mouse hybridoma cells was from Amano Pharmaceutical Co., Ltd, Japan (Tanaka *et al.*, 1989). Anti-human IGF-II polyclonal antisera (rabbit) was obtained from GroPep Pty. Ltd, Australia.

2.1.1.2.3 *IGF Binding Proteins -2, -3, -4 and -5*

All IGF binding proteins were polyclonal antisera (rabbit) purchased from Upstate Biotechnology Incorporated, New York, USA (anti-bovine IGF BP-2, anti-recombinant human IGF BP-3, anti-human IGF BP-4 and anti-chinese hamster ovary cell IGF BP-5).

2.1.1.2.4 *Other Antibodies*

Anti-human endothelial glycoprotein (100 kDa) monoclonal antisera derived from mouse hybridoma cells was from DAKO Corporation, California, USA. Horse anti-mouse IgG labelled with biotin was from Vector Laboratories Incorporated, California, USA. Goat anti-rabbit IgG labelled with biotin was obtained from Sigma Immunochemicals, Missouri, USA. Goat anti-rabbit IgG labelled with peroxidase was from Sigma Chemical Company, Missouri, USA.

2.1.1.3 Peptides and Hormones

The efficacy of all peptides derived from species other than sheep have been previously tested and shown to be suitable for use in sheep.

Human recombinant IGF-I and IGF-II were obtained from GroPep Pty. Ltd, Australia. Human recombinant IGF-I for the foetal infusion study was from Ciba Geigy, Switzerland. Ovine IGF-I (>99.99% pure) and ovine IGF-II (>99.99% pure) for receptor analyses were from Ciba Geigy, Switzerland (IGF-I) and Dr. Moore, AgResearch, New Zealand (IGF-II). Bovine insulin was from Sigma Chemical Company, Missouri, USA.

2.1.1.4 Enzymes

Boehringer Mannheim (Boehringer Mannheim NZ Ltd, NZ)

klenow polymerase; proteinase K; restriction enzymes; ribonuclease T1; T4 DNA ligase and 10 X ligase buffer

Promega Corporation (Dade Diagnostics Pty Ltd, NZ)

deoxyribonuclease I (ribonuclease-free); restriction enzymes; ribonuclease inhibitor; SP6 RNA polymerase; T7 RNA polymerase

Sigma Chemical Company (Missouri, USA)

ribonuclease A

2.1.1.5 Radioisotopes

Amersham International Plc (Life Sciences (NZ) Ltd, NZ)

[α-^{33}P]-labelled uridine-5'-triphosphate

[α-^{35}S]-labelled uridine-5'-triphosphate

[α-^{32}P]-labelled-2'-deoxy adenosine-5'-triphosphate

[α-^{32}P]-labelled-2'-deoxy cytosine-5'-triphosphate

New England Nuclear Ltd (Delaware, USA)

Na^{125}Iodide

2.1.1.6 Molecular Weight Markers

Lambda (cut with *Hind* III and *Eco* RI to yield: 21.226 Kb; 5.148 Kb; 4.973 Kb, 4.277 Kb; 3.530 Kb; 2.027 Kb; 1.904 Kb; 1.584 Kb; 1.330 Kb; 0.983 Kb; 0.564 Kb sized fragments) and an RNA ladder (7.4 Kb; 5.3 Kb; 2.8 Kb; 1.9 Kb; 1.6 Kb; 1.0 Kb; 0.6 Kb; 0.4 Kb; 0.3 Kb) were from Gibco BRL (Life Technologies Ltd, NZ).

A kaleidoscope pre-stained protein standard was purchased from Bio-Rad Laboratories, California, USA (myosin, blue, 208 kDa; β-galactosidase, magenta, 114 kDa; bovine serum albumin, green, 87 kDa; carbonic anhydrase, violet, 44.1 kDa; soybean trypsin inhibitor, orange, 32.7 kDa; lysozyme, red, 17.7 kDa; aprotinin, blue, 7.1 kDa)

A standard curve was constructed for each molecular weight marker, based on their relative mobility (Rf) (Rf = distance migrated by sample divided by the distance migrated by the dye front) and the log of their molecular weight. The relative mobility of each unknown sample (nucleic acid or protein) was determined from the standard curve.

2.1.1.7 Other Chemicals

Ajax Chemicals (Ajax Chemicals Ltd, NZ)

ethanol (bulk, analar); ethylenediamine tetra-acetic acid disodium salt (EDTA); magnesium chloride; saf solvent; xylene

Amersham International Plc (Life Sciences (NZ) Ltd, NZ)

enhanced chemiluminescence (ECL) detection kit; Hybond ECL nitrocellulose membrane; Hybond N nylon membrane

BDH Chemicals (BDH Chemicals New Zealand Ltd, NZ)

acetic acid; acetic anhydride; aluminium sulphate; ammonium sulphate; calcium chloride (fused granular 3-8 mesh); chloroform; chromium potassium sulphate; dichloromethane; dimethylsulphoxide; di-sodium hydrogen orthophosphate 2-hydrate; D.P.X mountant; eosin Y; ethanol (bulk, analar); ethylene glycol; formaldehyde; formamide (high and low grade); formic acid (analar, 90%); gelatine (solid sheet); glacial acetic acid; glucose; hydrochloric acid; hydrogen peroxide; iodine; isoamyl alcohol; lithium chloride; magnesium chloride; magnesium sulphate; methanol; methylene blue; N-ethylmaleimide (NEM); paraformaldehyde; paramat wax; phenol; potassium acetate; propan-2-ol; silica gel; sodium acetate; sodium azide; sodium chloride; sodium citrate; sodium di-hydrogen orthophosphate; sodium hydroxide; sodium iodate; sucrose; thymol crystals; toluene; trichloroacetic acid; triethanolamine; xylene cyanol

Beckman (Alphatech Systems Ltd, NZ)

liquid scintillation fluor

Biolab (Biolab Scientific Ltd, NZ)

0.45 μm millipore filters

Bio-Rad Laboratories (Bio-Rad Laboratories Pty Ltd, NZ)

alkaline copper tartrate; bis-acrylamide; bromophenol blue; coomassie brilliant blue; folin reagent; β-mercaptoethanol; AG-501-X8(D) mixed-bed resin; sodium dodecyl sulphate (SDS); Tween-20

Biotek (Biotek Ltd, NZ)

dynabeads kit

Boehringer Mannheim (Boehringer Mannheim NZ Ltd, NZ)

bovine serum albumin (BSA) (molecular biology grade, ribonuclease-free); dCTP; degraded herring sperm DNA; 1,4-dithiothreitol; dGTP; Mops; restriction enzyme buffers; Tris and Tris-HCl; tRNA from *Escherichia coli* strain HB101; dTTP

Bresatec Ltd (Australia)

Bresa-Clean™ nucleic acid purification kit

DAKO Corporation (Dimension Laboratories Inc., Canada)

wax pen (for immunohistochemistry)

Fluka BioChemika (Scianz Corporation, NZ)

guanidinium thiocyanate

FMC Corporation (Biolab Scientific Ltd, NZ)

high melting point agarose

George T. Gurr Ltd (England)

alcian blue

Gibco BRL (Life Technologies Ltd, NZ)

Escherichia coli (strain DH5α); guanidine hydrochloride; hexadeoxyribonucleotides; TRIzol®

Ilford (England)

K5 emulsion

J.T. Baker Chemical Company (Lab Supply Pierce (NZ) Ltd, NZ)

glycine

Kodak (Kodak New Zealand Ltd, NZ)

D-19 developer; Ilford Ilofix fixer; Kodak liquid X-ray developer Type 2; Kodak liquid X-ray fixer and replenisher; Kodak X-Omat™ AR film; NTB 2 nuclear track liquid emulsion

Local Suppliers

Ados F_2™ contact adhesive; halothane/oxygen for anaesthetising; liquid nitrogen; non-fat dried milk; normal goat serum; sodium pentobarbitone

May and Baker Australia PTY Ltd (Australia)

ammonium acetate; haematoxylin

Oxoid Ltd (Intermed Scientific Ltd, NZ)

phosphate-buffered saline tablets (PBS)

Pharmacia Biotech (Amrad Pharmacia Biotech Ltd, NZ)

cesium trifluoroacetate; sephadex G-50

Pierce Chemical Company (Illinois, USA)

dissucinimidyl suberate (DSS); iodogen

Promega (Dade Diagnostics Pty Ltd, NZ)

in vitro transcription kit containing: 5 X transcription buffer (200 mM Tris (pH 7.5); 30 mM $MgCl_2$; 10 mM spermidine), 1,4-dithiothreitol, CTP, GTP, ATP, UTP

Ruth Wagener Enterprises Inc. (Canada)

eukitt

Sigma Chemical Company (Missouri, USA)

3-aminopropyl triethoxysilane; ampicillin; avidin-biotin peroxidase (ABC complex); bacitracin; bovine γ globulin; bovine serum albumin (BSA) (96-99% pure); cesium trifluoroacetate; dextran sulphate; diaminobenzidine (DAB); diethyl pyrocarbonate; ethidium bromide; Ficoll (type 400); HEPES; N-lauryl sarcosine; β-mercaptoethanol; phenylmethyl sulphonyl fluoride (PMSF); polyethylene glycol (MW 8000); polypep; ponceau S; spermidine; Tris and Tris-HCl; Triton X-100

Tissue Tek, Miles Laboratories (Indianapolis, USA)

O.C.T. embedding medium

Waters Chromatography Division (Massachusetts, USA)

Sep-Pak C_{18} cartridge

2.1.2 Composition of Solutions

Unless otherwise stated, the composition of solutions were sourced from the standard molecular biology laboratory manuals (Sambrook *et al.*, 1989; Maniatis *et al.*, 1982), a standard histology text (such as Lillie, 1965) or from original papers describing the methodology for which they were used (see text for each method).

For all RNA analyses, equipment was treated with sterile milli-Q water containing 0.01% v/v diethyl pyrocarbonate. Solutions were treated overnight with 0.01% v/v diethyl pyrocarbonate followed by autoclaving as appropriate.

2.1.2.1 Buffers

Homogenisation Buffer (Barenton *et al.*, 1988)

(membrane preparation and cross-linking)

0.25 M sucrose, 1 mM PMSF; 0.02% w/v sodium azide; 1 mM N-ethylmaleimide (NEM)

Incubation Buffer (Elliott *et al.*, 1992)

(receptor analyses)

0.17 M Tris (pH 7.4); 1% w/v BSA, 5 mM $MgCl_2$; 0.002% w/v bacitracin; 1 mM PMSF (added just prior to use)

In Situ Hybridisation Buffer (Molenaar, 1995)

NaCl (150 mM); 2 x SSC; 50% v/v deionised formamide (salt and formamide concentrations were incorporated into the Tm calculation (Appendix); *Escherichia coli* tRNA (0.2 mg/ml) to prevent probe binding non-specifically to rRNA, tRNA or unidentified cellular components (Brahic and Haase, 1978; Lawrence and Singer, 1985); degraded herring sperm DNA (1 mg/ml), for the same reasons as tRNA (Brahic and Haase, 1978); ribonuclease-free bovine serum albumin (1 mg/ml) to saturate sites such as proteins, polysaccharides and nucleic acids in the sections that may have contributed to non-specific probe binding (Denhardt, 1966); 10% w/v dextran sulphate accelerates hybridisation rate by sequestering water, thereby increasing effective probe concentration (Wahl, *et al.*, 1979); 1,4-dithiothreitol (0.1 M) maintains a reducing environment to prevent irreversible non-specific ^{35}S binding (Lawrence and Singer, 1985; Singer *et al.*, 1987).

Northern Hybridisation Buffer (modified from Church and Gilbert, 1984)

500 mM $NaHPO_4$; 1 mM EDTA; 7% SDS (1 M $NaHPO_4$ (pH 7.2) stock is composed of 134 g of $Na_2HPO_4.7H_2O$ and 4 ml of 85% H_3PO_4 per liter)

Phosphate Buffered Saline (PBS) (pH 7.5)

11.5 g di-sodium hydrogen orthophosphate, anhydrous; 2.96 g sodium di-hydrogen orthophosphate; 5.84 g sodium chloride; milli-Q water to 1000 ml. pH 7.5 with HCl

Post Incubation Buffer (Elliott *et al.*, 1992)

(receptor analyses)

0.17 M Tris (pH 7.4); 0.25% w/v BSA; 0.1% v/v Triton X-100

Protein Transfer Buffer (Laemmli, 1970)

25 mM Tris (pH 8.3); 0.2 M glycine; 20% methanol

5 X Random Primed Labelling Buffer

0.25 M HEPES, pH 6.6; 25 mM $MgCl_2$; 50 mM β-mercaptoethanol; 2 mg/ml BSA; (1 mM dCTP; 1 mM dGTP; 1 mM dTTP) if using labelled dATP or (1 mM dGTP; 1 mM dTTP; 1 mM dATP) if using labelled dCTP

Sodium Phosphate Buffer (1 M stock)

9.5% w/v 2 M sodium di-hydrogen orthophosphate (NaH_2PO_4); 40.5% w/v 2 M di-sodium hydrogen orthophosphate (Na_2HPO_4); pH 7.4

Storage Buffer (Barenton *et al.*, 1988)

(membrane preparation and cross-linking)

25 mM Tris (pH 7.4); 10 mM $MgCl_2$ 1 mM PMSF; 1 mM NEM

TE Buffers (Davis *et al.*, 1986)

pH 7.6 (10 mM Tris [pH 7.6]; 1 mM EDTA [pH 8.0])

pH 8.0 (10 mM Tris [pH 8.0];1 mM EDTA [pH 8.0])

5 X Transcription Buffer (Promega Corporation, 1990)

200 mM Tris (pH 7.5); 30 mM Mg Cl_2; 10 mM spermidine. Spermidine is a polyamine that has been shown to stimulate RNA polymerases (Blair, 1986; Jain and Tyagi, 1987)

Tris-buffered Saline (TBS)

0.15 M NaCl; 0.01 M Tris; pH 7.6

Tris Incubation Buffer (Barenton *et al.*, 1988)

(membrane preparation and cross-linking)

25 mM Tris (pH 7.4); 10 mM $MgCl_2$; 0.1% w/v BSA

2.1.2.2 Other Solutions

Coomassie Blue Staining Solution (Deutscher, 1990)

0.25% w/v coomassie brilliant blue; 50% methanol; 10% v/v acetic acid

DAB (diaminobenzidine)

One DAB tablet was dissolved in 15 ml of 50 mM Tris (pH 7.6), filtered through Whatman filter paper, and immediately before use, 12 µl 30% v/v hydrogen peroxide was added.

Deionised Formamide

Molecular Biology grade formamide (500 ml) was deionised in 25 g AG-501-X8(D) mixed-bed resin for 30 minutes. After filtering, aliquots were stored at -20°C

10% Neutral-Buffered Formalin (Humason, 1979)

10% v/v formaldehyde; phosphate-buffered saline tablets (1 tablet per 100 ml = 33 mM sodium di-hydrogen orthophosphate [$NaH_2PO_4.H_2O$]; 46 mM di-sodium hydrogen orthophosphate [Na_2HPO_4]). The mixture was made using sterile, diethyl pyrocarbonate-treated Milli-Q water.

4% Paraformaldehyde

4% v/v paraformaldehyde; phosphate-buffered saline tablets (1 tablet per 100 ml = 33 mM sodium acid phosphate [$NaH_2PO_4.H_2O$]; 46 mM anhydrous disodium phosphate [Na_2HPO_4]). The mixture was made using sterile, diethyl pyrocarbonate-treated Milli-Q water.

Ponceau S (Salinovich and Montelaro, 1986)

0.1% w/v ponceau S; 1% v/v acetic acid

20 X SSC (Davis *et al.*, 1986)

3 M sodium chloride; 0.3 M sodium citrate (pH 7.0 with sodium hydroxide). From 20 X SSC stock, appropriate dilutions of 10 X SSC, 3 X SSC, 2 X SSC and 0.2 X SSC were made.

2.2 METHODS

2.2.1 Tissue Collection

2.2.1.1 **Ethics and Radiation Safety**

Prior consent for all animal manipulations was obtained from the Ruakura Animal Ethics Committee and the University of Waikato Animal Ethics Committee.

The ^{125}I tracer study (Chapter 6) was carried out with the guidance and approval of the Ruakura Radiation Safety Officer.

2.2.1.2 **Animals**

2.2.1.2.1 *Foetal Ontogeny Study* (Chapters 4 and 5)

A total of 250 mixed age ewes were mated to 4 rams at behavioural oestrus (day 0 of gestation) to generate 72 foetal Coopworth X Dorset animals for sampling. At 40 days gestation ewes were pregnancy-tested by ultrasound scanning. On the basis of these results, 36 pregnant animals thought to be carrying twins were divided into 6 slaughter groups balanced for ewe weight at 49 days gestation and ram usage across the groups. Sampling of foetal tissue was carried out at 50, 62, 82, 103, 124 and 140 days gestation. These ages were chosen to evenly span sheep foetal bone development.

Long bones were collected from either 10, 11 or 12 foetal lambs per gestational age depending on actual twinning status. Right hind limbs were processed for histological analyses and right fore limbs were frozen to enable extraction of RNA, protein or membranes as appropriate. Right forelimbs from some animals at 62, 103 and 140 days gestation were frozen, sectioned and analysed for IGF binding sites. Left hind limbs from 50 days gestation were used to test the effect of decalcifying time on the ability to detect mRNA and protein (Chapter 3).

2.2.1.2.2 *Foetal Infusion of labelled IGF-I* (Chapter 6)

Mixed age ewes were mated to two rams at behavioural oestrus, to provide Coopworth X Dorset foetuses of known gestational age for this study. Foetal surgery was performed on six ewes between 133 and 140 days gestation carrying single foetuses (determined by ultrasound scanning at 70 days gestation). Each ewe was anaesthetised with sodium pentobarbitone (20 mg/kg) with maintenance on halothane/oxygen. After anaesthetisation, the foetus was exposed *in utero* and a bilateral vascular catheter was inserted into the foetal carotid artery. The catheters were flushed frequently with saline to maintain patency. This allowed delivery of ^{125}I-labelled IGF-I and sampling of foetal blood. An additional catheter was inserted into amniotic fluid.

All foetuses received a 1 ml bolus of ^{125}I-labelled IGF-I (~70 μCi; ~550 ng IGF-I, diluted to 1 ml in 0.25% w/v BSA/PBS), following which, the catheter was immediately flushed with physiological saline (section 2.2.2.2). Three animals were sacrificed after 12 minutes while the remaining three animals were sacrificed after 180 minutes following injection of ^{125}I-labelled IGF-I. Samples of foetal blood (1 ml) were withdrawn from the contralateral catheter at 1 and 10 minutes (sacrifice at 12 minutes) or 1, 2, 5, 10, 20, 60 and 180 minutes (sacrifice at 180 minutes). Amniotic fluid was sampled at 10, 60 and 180 minutes and one sample of maternal blood was withdrawn at 180 minutes after infusion. All fluid samples were withdrawn into glass blood collection tubes containing EDTA and immediately chilled on ice. Plasma was separated from whole blood by centrifugation at 1500 X g for 15 minutes at 4°C and processed as described (section 2.2.3.6). The proximal femur, distal tibia, proximal humerus and distal radius were collected from all animals and processed as described below. A sample of liver, kidney and skeletal muscle from two animals (sacrifice at 12 minutes and sacrifice at 180 minutes) were also collected as reference tissues to bone.

2.2.1.2.3 *Postnatal Ontogeny Study* (Chapters 3 and 5)

Long bones from Coopworth X Dorset lambs aged 2 days, 2 weeks, 6 months and 18 months were sampled. Initially, samples were taken from one animal at each age. In an attempt to overcome technical difficulties associated with processing postnatal bones, additional bone samples from one animal at each age were collected. Right hind limbs were processed for histological analyses and right forelimbs were frozen for RNA extraction.

2.2.1.2.4 *Additional Animals* (Chapters 3, 4, and 5)

Long bones were collected from two foetal sheep at approximately 100 and 124 days gestation. This was a pilot study to determine appropriate tissue processing methods for the ontogeny studies. Rat tissues from both foetal and postnatal development were collected to serve as controls where required. Additional sheep tissues from previous work in this laboratory were used for controls where required.

2.2.1.3 Tissue Collection and Processing

Immediately following death, bone was dissected free from the animal, and soft tissue trimmed as close as possible to the bone. Whole legs were collected from animals at 50, 62

and 82 days gestation, as no further dissection was practical. After 82 days gestation, individual bones were dissected. To facilitate fixation or freezing, bones from 124 days gestation and older were split longitudinally using a fret saw. For a particular bone, the cutting plane was the same between all animals.

2.2.1.3.1 *Histological Methods*

Bones and other soft tissues required for histological analyses (except the receptor binding assay) were fixed in 10% neutral-buffered formalin for 24 hours at 4°C. Following fixation, bones were immersed in 5% v/v formic acid to decalcify them sufficiently for sectioning (Mullink *et al.*, 1985; Chapter 3). The length of time for decalcification was determined empirically for each age group and was carried out at 4°C (see Chapter 3). Bones from 50, 62 and 82 days gestation were decalcified for 1 day, bones from 103 days gestation were decalcified for 7 days, whereas all older bones were decalcified for 20 days.

Following decalcification, each bone was microdissected to produce an appropriate size and representation of bone, including the growth plate and diaphysis. In samples taken from 50 and 62 days gestation, the whole leg was represented in a single section. As the samples became larger with gestation, first individual bones and then particular areas of bone were represented in each tissue block. All samples were orientated within the block to present a longitudinal plane for sectioning through bone.

Tissue was embedded in an automated Shandon Elliott Duplex processor according to standard processing protocols (Humason, 1979). The procedure was as follows: dehydration at room temperature (70% ethanol, 1 hour; 96% ethanol, 1 hour; 96% ethanol, 1 hour; 96% ethanol, 1 hour; 99% ethanol, 1 hour; 99% ethanol, 1.5 hour; 99% ethanol, 1.5 hour) followed by clearing at room temperature (toluene, 1 hour; toluene, 1 hour) and impregnation at 60°C (paraffin wax, 3 hours; paraffin wax, 3 hours). Tissues were removed from the processor, placed into moulds and the moulds filled with molten (60°C) paraffin wax. The blocks were set at room temperature before sectioning.

Sections, 7 μm thick, were cut on a microtome. Sections were heat-stretched in a 60°C water bath to remove wrinkles prior to mounting. Two adjacent sections or individual sections (as appropriate) were floated on to pre-cleaned aminoalkylated glass slides. The slides were air dried and then baked for 24 hours at 45°C.

2.2.1.3.2 *Extraction Methods and Cryosections*

Bone and soft tissues required for extraction of RNA, protein, membranes or calcified sections, were snap frozen in liquid nitrogen. They were stored at -80°C until required. Frozen bone samples were microdissected from two areas - the diaphysis (including the periosteum) and the epiphyseal growth plate. Each bone was mounted using O.C.T. embedding medium on the cooling block of a sledge microtome (-40°C) and cut in half longitudinally with a fine fret saw. All equipment and tissue was kept on dry ice during the procedure. Growth plate or diaphysis samples (approximately 100 mg) were then cut from the appropriate area (being careful to exclude skeletal muscle) and extracted to yield cell membranes, RNA or protein.

As appropriate, calcified sections were cut from frozen bone using a sledge microtome at -16 to -20°C. Sections (10 μm) were thaw-mounted onto aminoalkylated glass slides for receptor binding assays or for the *in situ* control experiment (Chapter 3). Cryosections for *in situ* hybridisation were fixed in fresh 4% paraformaldehyde for 20 minutes and then dehydrated (3 X phosphate-buffered saline (PBS), 5 minutes; 1 X PBS, 5 minutes; 1 X PBS, 5 minutes; 30% ethanol, 2 minutes; 50% ethanol, 2 minutes; 70% ethanol, 2 minutes; 96% ethanol, 2 minutes; 96% ethanol, 2 minutes). All slides were air dried and stored at -70°C with desiccant until use.

2.2.2 Radioactive Tracers

2.2.2.1 **Probe Synthesis**

The method of probe generation and the choice of radioactive label depended on the intended use. Single-stranded RNA probes (riboprobes) were used for *in situ* hybridisation whereas DNA probes were generated for Northern analysis.

RNA probes offer several advantages for *in situ* hybridisation. Higher washing temperatures can be used because cRNA-mRNA hybrids are more stable than corresponding cDNA-mRNA hybrids. RNA probes are single-stranded therefore the complementary strand cannot compete with mRNA binding and ribonucleases can be used to remove unhybridised probe (Pintar and Lugo, 1987; Cox *et al.*, 1984). Riboprobes were labelled with ^{35}S since this isotope met the requirements for sensitivity (emission energy is 0.167 MeV), anatomical resolution (10-15 μm) and speed of development (Tecott *et al.*, 1987; Penschow, 1990). For the detection of rare messages, ^{33}P was incorporated into RNA probes and used for *in situ* hybridisation. Greater sensitivity can be achieved with ^{33}P since it has a higher emission energy (0.294 MeV). However, for most experiments ^{35}S was used since ^{33}P has a shorter half-life, lower resolution and is more costly.

DNA probes for Northern analysis were generated by random priming. They offer the following advantages. Templates for DNA probes are easier to generate, less care is needed to prevent probe degradation compared to RNA probes and they can be labelled to high specific activity (Feinberg and Vogelstein, 1983). Probes were labelled with ^{32}P since its high emission energy (1.710 MeV) offers good sensitivity (Penschow, 1990).

2.2.2.1.1 *DNA Probes - Random Primer Extension*

DNA probes were synthesized by random primer extension originally described by Feinberg and Vogelstein (1983; 1984). In this method a mixture of all possible denatured hexanucleotides is hybridised to a denatured DNA template, klenow (large fragment of *Escherichia coli* DNA polymerase I) is then added which utilises the hexanucleotides as a primer for synthesis of DNA from the complementary strand.

A random priming procedure adapted from an original method was followed (Mackey *et al.*, 1992). DNA templates were excised from their plasmids with appropriate restriction enzymes and size-fractionated on a 1% w/v agarose gel. Ethidium bromide was included in the DNA sample and fluorescence of ethidium bromide complexed to DNA was visualised by ultraviolet illumination. The appropriate DNA band was excised from agarose and stored at -

20°C. Prior to probe generation, template DNA was heated to 65°C for 5 minutes to melt the agarose. Approximately 25 ng template DNA, 3 µg of a random hexanucleotide mix and TE buffer (pH 8.0) to a final volume of 30 µl were boiled for 10 minutes. After rapid cooling on ice, 10 µl 5 X labelling mix, 5 µl (50 µCi) of either adenosine 5′-[α-^{32}P]triphosphate or cytidine 5′-[α-^{32}P]triphosphate and 5 U Klenow enzyme were added. The mixture was incubated at 37°C for approximately 45 minutes after which the volume was increased to 100 µl by the addition of TE buffer (pH 8.0). Probe was purified from unincorporated labelled deoxynucleotide triphosphates by centrifugation through a Sephadex G-50 column equilibrated with TE buffer (pH 8.0) (Maniatis *et al.*, 1982). Labelling efficiency ranged from 30 - 50% (ratio of radioactivity measured in a liquid scintillation counter in 1 µl sampled before column purification to radioactivity in 1 µl sampled after column purification). Typically, random primed labelled DNA probes had a specific activity of 1 - 1.5 X 10^9 dpm/µg.

2.2.2.1.2 *RNA Probes - Riboprobe Runoff Transcription*

RNA probes were synthesised from the appropriate template according to a protocol developed by Melton *et al.* (1994), and specified by Promega Biotec (Promega Corporation, 1990).

Linearisation of plasmid

Recombinant pGEM 3 plasmids containing cDNA templates were each linearised with appropriate restriction enzymes adjacent to the cDNA template, resulting in either an SP6 or T7 promoter upstream and a cut end downstream of the template. This enabled run-off transcripts to be generated that contained only template sequences (i.e. no vector sequences). Restriction enzymes were chosen that do not yield 3′ protruding ends since 3′ protruding ends can cause re-priming of transcription along the opposite strand (Schenborn and Mierendorf, 1985). Agarose gel electrophoresis was carried out to check if the DNA had been completely linearised. Incomplete digestion affects transcript purity (due to the presence of vector sequences) and yield (incorporation of uridine 5′-(α-[^{35}S]thio) triphosphate into unwanted vector sequences) (Promega Corporation, 1990). After linearisation, DNA was ethanol precipitated and resuspended in TE buffer (pH 7.4) at 200 ng/µl.

Transcript Synthesis

At room temperature, the following (in order) were added to two Eppendorf tubes and mixed: 4.0 µl 5 X transcription buffer; 2.0 µl 100 mM 1,4-dithiothreitol (provided reducing conditions for the ^{35}S label to help prevent non-specific binding) (Angerer *et al.*, 1987); 1.3 µl 2.5 mM CTP; 1.3 µl 2.5 mM GTP; 1.3 µl 2.5 mM ATP; 0.8 µl ribonuclease inhibitor (25 U/µl) (Blackburn *et al.*, 1977); 50 µCi (5 µl) uridine 5´-(α-[^{35}S]thio) triphosphate and diethyl pyrocarbonate-treated Milli-Q water, to a final volume of 21 µl. To one tube was added 0.2 - 1.0 µg linearised template DNA (restricted to generate the T7 promoter upstream from template DNA) and 0.5 µl T7 RNA polymerase (19 U/µl). To the other tube was added 0.2 - 1.0 µg linearised plasmid DNA (restricted to generate the SP6 promoter upstream from template DNA) and 0.5 µl SP6 RNA polymerase (15 U/µl). The transcription mixture was incubated for 60 minutes at 37°C. Labelling efficiency was tested by trichloroacetic acid precipitation. Typical incorporations ranged from 40 - 95%.

DNA template was removed from the transcription mixture by treatment with ribonuclease-free deoxyribonuclease I to prevent template annealing to probe and/or target during hybridisation (Angerer *et al.*, 1987). Ribonuclease-free deoxyribonuclease (1 U/µl) was added to the transcription mixture and incubated at 37°C for 20 minutes. The RNA probe was ethanol precipitated to remove unincorporated nucleotides and resuspended in 21 µl 10 mM 1,4-dithiothreitol. A 1 µl sample of probe was removed to measure radioactivity in a liquid scintillation counter. Typical activities ranged from 0.4 - 1.0 X 10^6 cpm/µl. This resulted in riboprobes with specific activities in the range 0.6 - 4.0 X 10^7 cpm/µg.

2.2.2.2 Radioiodination of IGF-I and IGF-II

Radioiodinated IGF-I and IGF-II were used as tracers in receptor binding and affinity cross-linking assays (Chapter 4). Radioiodinated IGF-I was also used *in vivo* to study the movement of IGF-I from the circulation into bone (Chapter 6).

IGF-I and IGF-II were radioiodinated using a modification of the Iodogen method initially described by Salacinski, McLean *et al.* (1981). This method generates labelled proteins with

high specific activity and stability whilst maintaining physiochemical integrity of the protein being labelled. The integrity of IGF-I and IGF-II has previously been established using the Iodogen method in this laboratory (Hodgkinson, 1991a).

Iodogen (16 µg in 200 µl dichloromethane) was placed in a tube and the solvent evaporated. To this was mixed 20 µl of 0.064 M phosphate buffer (pH 7.4), 60 µl IGF-I or IGF-II (approximately 3.8 µg) and approximately 5 µl Na ^{125}Iodide (0.5 mCi; corrected for decay) to give a final volume of 85 µl. Iodination was allowed to continue for 10 minutes at room temperature after which 50 µl 0.064 M phosphate buffer (pH 7.4) was added. After a further 5 minutes incubation, 365 µl 0.1% w/v Polypep in a solution of 0.01 N HCl; 0.15 M NaCl were added. Unincorporated Na^{125}I was separated from ^{125}I-labelled IGF-I or IGF-II by chromatography using a Sep-Pak C_{18} cartridge pre-equilibrated with a solution containing 0.01 N HCl; 0.15 M NaCl. After the iodination mixture was loaded onto the column, the column was washed with a solution of 0.01 N HCl; 0.15 M NaCl to elute unincorporated radioiodine. Adsorbed peptide was eluted in 2 ml fractions using a step-wise gradient of ethanol made up in a solution of 0.01 N HCl; 0.15 M NaCl. Radioactivity in a sample of each fraction was measured in a gamma scintillation counter. The incorporation of iodine ranged from 82 - 97% resulting in specific activities of 103 - 122 µCi/µg. The fractions eluted in 40 - 45% ethanol; 0.01 N HCl; 0.15 M NaCl corresponded to ^{125}I labelled IGF. These fractions were stored at -20°C and used for all studies.

2.2.3 Assays

2.2.3.1 *In situ* Hybridisation

2.2.3.1.1 *Introduction*

In situ hybridisation is a technique that allows RNA of interest to be localised at the cellular level within a tissue. Detection of RNA is achieved with high specificity and sensitivity. The first procedure for *in situ* hybridisation was published in 1969 (Gall and Pardue, 1969; John *et al.*, 1969). RNA was isolated, labelled and incubated with tissue squashes or cultured cells where it hybridised to denatured complementary DNA. Since then, the technique has evolved

to enable detection of viral DNA sequences, gene mapping of chromosomes and mRNA with appropriate probes. The present work used *in situ* hybridisation to study the localisation of RNA using RNA probes.

2.2.3.1.2 *Section Pre-treatment*

Sections were deparaffinised by passing the slides through three changes of xylene for 10 minutes each. Rehydration was through an alcohol series of decreasing ethanol concentrations (96%, 96%, 95%, 70%) for 5 minutes each. The slides were rinsed in diethyl pyrocarbonate-treated Milli-Q water for 2 minutes.

Mild deproteination with acid followed by enzyme treatment was carried out to increase permeability of probe and remove proteins from target RNA. Slides were placed in 0.2 N hydrochloric acid for 10 minutes at room temperature to remove basic proteins (Brahic and Haase, 1978; Gee and Roberts, 1983). After 30 minutes at room temperature in 2 X SSC buffer, sections were treated with protease. A mixture of 200 mM Tris (pH 2.7) and 2 mM $CaCl_2$ was heated to 37°C and proteinase K (30 U/µl) was added (Ebeling *et al.*, 1974). Slides were incubated in the proteinase K solution for 15 minutes at 37°C (Brahic and Haase, 1978; Angerer and Angerer, 1981; Gee and Roberts, 1983).

Acetylation of amino groups was carried out to reduce non-specific electrostatic binding of the probe (Hayashi *et al.*, 1978). To 0.1 M triethanolamine (pH 8.0), 0.25% v/v acetic anhydride was added, mixed thoroughly and immediately poured over the slides. After 5 minutes, a further 0.25% v/v acetic anhydride was added and incubation carried out a further 5 minutes at room temperature. Sections were washed for 5 minutes in 2 X SSC buffer to remove traces of acetic anhydride. Dehydration was through an alcohol series of increasing ethanol concentrations (70%, 95%, 96%) for 5 minutes each. The slides were then air-dried. A well to hold the hybridisation buffer was formed by applying a ring of contact adhesive around each section.

2.2.3.1.3 *Hybridisation*

The appropriate probe was diluted in *in situ* hybridisation buffer to give a concentration of 30 000 cpm/µl (approximately 0.7 ng/µl). This concentration was determined empirically

(Hodges, 1992). Approximately 15 µl/10 mm^2 of the appropriate probe was applied to each section. On each slide a pair of adjacent sections were mounted. One section was incubated with antisense probe and the other section was incubated with sense probe. Hybridisation was carried out overnight (15 - 20 hours) at the appropriate hybridisation temperature (generally 58°C, determined empirically based on the Tm calculation for each probe, see Appendix) in a humidified chamber equilibrated with 50% v/v formamide, 2 X SSC.

2.2.3.1.4 *Post-Hybridisation Washes*

The washing procedure was determined empirically, based on stringency calculations (Tm) and procedures adopted by others (Appendix, Hodges, 1992; Valentino *et al.*, 1987; Polak and McGee, 1990).

Excess probe was removed from sections by washing twice (5 and 15 minutes) in a pre-warmed solution of 2 X SSC buffer containing 50% v/v formamide and 10 mM β-mercaptoethanol (Brandtlow *et al.*, 1987; Shivers *et al.*, 1989). These washes were carried out at the same temperature as hybridisation. The slides were then washed with agitation in four changes of 2 X SSC buffer containing 10 mM β-mercaptoethanol for 15 minutes each at room temperature. The contact adhesive was removed from each slide.

Non-specific binding of probe was further reduced by exploiting the specificity of ribonuclease A and ribonuclease T1 for single-stranded RNA (John *et al.*, 1969; Cox *et al.*, 1984). Slides were incubated for 30 minutes at 37°C in pre-heated 2 X SSC buffer (37°C) containing ribonuclease T1 (3.5 U/ml) and ribonuclease A (0.07 U/ml). Washing was continued in 2 X SSC buffer with 10 mM β-mercaptoethanol for 10 minutes, then three times in 0.2 X SSC buffer with 10 mM β-mercaptoethanol for 5 minutes each. Dehydration was through an alcohol series of increasing ethanol concentrations (70% with 10 mM β-mercaptoethanol, 80%, 96%) for 5 minutes each.

2.2.3.1.5 *Autoradiography*

After drying, sections were exposed in an X-ray cassette at room temperature to X-ray film which provided a rapid method for determining tissue background and strength of hybridisation signal (Hudson *et al.*, 1981). Exposure periods were determined empirically for

each *in situ* hybridisation experiment and ranged from four hours (human ribosomal RNA) to seven days (ovine Type I IGF receptor). The X-ray films were developed in an automatic film processor.

Coating sections with liquid emulsion enabled the microscopic resolution of probe binding. Each section was coated with liquid emulsion and exposed (section 2.2.3.7.1). Exposure periods ranged from 7 days (human ribosomal RNA) to 70 days (ovine Type I IGF receptor). Sections were lightly stained with haematoxylin and eosin and mounted under coverslips with D.P.X. (Appendix).

2.2.3.2 Northern Analysis

2.2.3.2.1 *Introduction*

Northern hybridisation is a procedure that determines the size of RNA of interest. It involves isolation of RNA, size-fractionation of RNA on a denaturing agarose gel, transfer to a membrane support and hybridisation with a labelled, complementary probe (Alwine *et al.*, 1977).

2.2.3.2.2 *RNA Extraction*

For each RNA extraction, frozen, dissected tissue ranging from 0.1 - 2 g was either pulverised to a powder manually in a mortar containing liquid nitrogen or ground to a powder in a tissue compactor containing liquid nitrogen.

RNA extraction - Method 1 (soft tissues)

RNA was extracted according to a protocol supplied with TRIzol® (Life Technologies, 1995) based on the single-step RNA isolation procedure published by Chomczynski and Sacchi (1987). Powdered tissue was homogenised in TRIzol® reagent (1 ml TRIzol® per 100 mg tissue) and after incubation at room temperature for 5 minutes, 0.2 ml of chloroform per 1 ml starting volume was added. The mixture was mixed vigorously for 3 minutes and then centrifuged at 12 000 g for 15 minutes at 4°C. The aqueous phase containing RNA was separated from the lower red, phenol-chloroform phase into a fresh tube. The RNA was precipitated with propan-2-ol (0.5 ml per 1 ml starting volume) and following 10 minutes

incubation at room temperature, was pelleted by centrifugation at 12 000 g for 10 minutes at 4°C. The pellet was washed in 75% ethanol and the RNA resuspended in TE buffer, pH 7.6.

RNA extraction - Method 2 (skeletal tissue)

Powdered tissue was further homogenised manually in 3 ml of 4 M guanidinium thiocyanate using a ground glass mortar and pestle and 300 µl of 10% w/v sarcosyl was then added (Chomczynski and Sacchi, 1987). The mixture was then centrifuged at 7 650 g for 2 hours at 18°C and the supernatant was filtered through 0.45 µm mesh in a millipore apparatus (Nemeth *et al.*, 1989). This was followed by acidification and phenol-chloroform extraction (Chomczynski and Sacchi, 1987). To the filtered supernatant, 0.3 ml 2 M sodium acetate (pH 4), 3 ml phenol (water-saturated) and 0.6 ml chloroform-isoamyl alcohol mixture (49:1) were added sequentially. The mixture was shaken vigorously and cooled on ice for 15 minutes. The suspension was then centrifuged at 10 000 g for 20 minutes at 4°C. The aqueous phase was transferred to a fresh tube and prepared for centrifugation in cesium trifluoroacetate (Zarlenga and Gamble, 1987).

Cesium trifluoroacetate was added to give a final concentration of 1.34 g/ml. This resulted in a gradient in the range 1:1.6 to 1:0.6 (homogenate:cesium trifluoroacetate). The mixture was spun at 150 000 g at 15°C for 20 hours in a SW 40.Ti rotor. On some occasions an RNA band was present halfway up the gradient, whereas on other occasions RNA was found as a precipitate at the bottom of the tube. The RNA was washed three times with 70% ethanol and redissolved in TE buffer, pH 7.6.

After determining RNA yield using a UV absorption spectrophotometer, RNA was ethanol precipitated and resuspended at 1 µg/µl in deionised formamide. The yield of total RNA was generally between 50 and 100 µg/100 mg tissue. RNA preparations had a ratio of absorbance at λ 260 nm to absorbance at λ 280 nm of between 1.4 and 2.0 indicating relatively low protein contamination. RNA was stored at -70°C in deionised formamide until required.

2.2.3.2.3 *Northern Hybridisation*

RNA samples were electrophoretically separated on a 1.2% w/v denaturing agarose gel (Appendix). Depending on the mRNA of interest, between 10 µg and 50 µg total RNA was

loaded on each gel. After separation was complete, the samples were transferred to Hybond N® nylon membrane by capillary action in a standard blotting procedure (Maniatis *et al.*, 1982). Blotting was carried out overnight in 10 X SSC buffer. RNA was then covalently fixed to the membrane by exposure to ultraviolet radiation (254 nm) in a Stratalinker UV crosslinker. A standard delivery of 120 000 μJ was used for optimal fixation of RNA.

In order to evaluate the quality and quantity of RNA, the membrane was stained in methylene blue (0.02% w/v methylene blue; 0.3 M sodium acetate, pH 5.5) for approximately 10 minutes (Herrin and Schmidt, 1988). Following staining, the membrane was destained in milli-Q water, photographed and photocopied. Prior to pre-hybridisation, the stain was removed from the RNA and membrane by washing in 3 X SSC, 0.1% w/v SDS, for 15 minutes.

2.2.3.2.4 *Hybridisation and Washing*

Pre-hybridisation of the membrane in 5 ml of Northern hybridisation buffer was carried out in a hybridisation oven for a minimum of 1 hour at 65°C. DNA probes were denatured immediately prior to use by either the addition of sodium hydroxide to a final concentration of 0.3 M or boiling for 10 minutes. Denatured probe was added to the membrane and allowed to hybridise overnight at 65°C (unless otherwise indicated). Post hybridisation washing consisted of three changes in 0.1% w/v SDS, 3 X SSC. The first wash was at 65°C, while subsequent washes were at room temperature with agitation.

2.2.3.2.5 *Autoradiography*

The Northern blot was exposed to X-ray film for between 4 hours and 14 days at -80°C in an X-ray cassette containing intensifying screens (Hahn, 1983; Laskey and Mills, 1977; Swanstrom and Shank, 1978). X-ray film was developed in an automatic film processor.

2.2.3.3 Receptor Analyses

2.2.3.3.1 *Introduction*

Potential IGF-I and IGF-II binding sites were localised by a ligand binding assay. Further characterisation of these binding sites was achieved by affinity cross-linking IGF-I or IGF-II to a crude microsomal extract.

2.2.3.3.2 *Ligand Binding Assay*

Calcified bone cryosections (section 2.2.1.3.2) were stored at -70°C with desiccant and used within one week of cutting. *In vitro* receptor incubations were carried out using a modification of the procedure developed by Maurer and Reubi (1986) and described by Elliott *et al.* (1992). In each pair of adjacent sections, one section was incubated with either ^{125}I-IGF-I or ^{125}I-IGF-II to determine total binding sites for IGF-I or IGF-II, respectively. The other section served as a control to determine non-specific or residual binding. This section was incubated with either a mixture of ^{125}I-IGF and an excess of unlabelled homologous IGF (1 µg/ml) or ^{125}I-IGF and physiological concentrations of unlabelled insulin (10 ng/ml). Specific binding represents the difference between total binding and non-specific or residual binding.

While sections were thawing, a ring of contact adhesive was made around each section to form a well. Sections were treated with 0.17 M Tris (pH 7.4) for 10 minutes at room temperature. This was replaced with 50 µl incubation buffer containing the appropriate iodinated hormone to give a concentration of 400 cpm/µl (approximately 1.6 ng/µl). Sections were incubated for two hours at room temperature in an humidified chamber equilibrated with water. Slides were then washed twice for 5 minutes each in ice-cold post-incubation buffer followed by fixation in 10% neutral-buffered formalin for 10 minutes. A final wash was carried out in distilled water for 5 minutes.

Autoradiography

After drying, sections were exposed to X-ray film in the same way as for *in situ* hybridisation (section 2.2.3.1.5). Appropriate exposures were determined empirically for each experiment. Typical exposures were for 3 days. Sections were then coated with liquid emulsion to determine cellular localisation of ^{125}I-IGF binding (section 2.2.3.7.1). Exposures were from 14 days (IGF-II binding) to 21 days (IGF-I binding). Sections were lightly counterstained with alcian blue followed by haematoxylin and eosin and mounted under coverslips in D.P.X. (Appendix).

2.2.3.3.3 *Binding Site Characterisation Microsomal Membrane Preparation*

A preparation of crude microsomal membranes from a microdissected growth plate was extracted following the method of Barenton *et al.* (1988) and analysed for IGF-I and IGF-II

binding sites. Tissue (1.2 g) was manually crushed to a powder in liquid nitrogen using a mortar and pestle and then homogenised in 5 ml ice-cold homogenisation buffer using an Ultra-Turrax on high speed for 2 periods of 15 seconds. This was followed by centrifugation at 1 300 g for 20 minutes at 4°C. The supernatant was kept on ice while the pellet was resuspended in 2 ml of homogenisation buffer and respun at 1 300 g for 20 minutes at 4°C. The second supernatant was pooled with the supernatant from the first spin. The pooled supernatants were then spun at 100 000 g for 60 minutes at 4°C. The pellet containing the microsomal fraction (plasma, nuclear and intracellular membranes) was resuspended in 1 ml of storage buffer. To further wash the fraction, it was respun at 100 000 g for 60 minutes and the pellet resuspended in 1 ml storage buffer. The crude microsomal membrane fraction was kept at -20°C until use.

Affinity Cross-linking

Labelled IGF-I or IGF-II (^{125}I-IGF-I or ^{125}I-IGF-II) was affinity cross-linked to protein in the crude microsomal membrane fraction. This was performed according to a modification of the method of Barenton *et al.* (1988). Membrane samples (100 µg protein) were incubated at 4°C overnight in Tris incubation buffer with either ^{125}I-labelled IGF-I or ^{125}I-labelled IGF-II (1 X 10^6 cpm) with and without excess unlabelled homologous hormone (1 µg/ml). The following day, 1 ml of bovine γ-globulin (0.125% w/v in 25 mM Tris (pH 7.4); 10 mM $MgCl_2$) and 1 ml 25% w/v polyethylene glycol (PEG 8000) in 25 mM Tris (pH 7.4) were added to each sample. The samples were then mixed and incubated at 4°C for 15 minutes, following which they were centrifuged at 4 000 g for 30 minutes at 4°C. Pellets were resuspended in 100 µl of 0.064 M phosphate buffer. Dissucinimidyl suberate was freshly dissolved in dimethylsulphoxide and added to each sample at a final concentration of 0.35 mM. After mixing, samples were incubated on ice for 15 minutes, following which 60 µl 1 M Tris (pH 6.8) was added. This was followed by centrifugation at 4 000 g for 30 minutes at 4°C. The pellets were resuspended in 50 µl of 0.064 M phosphate buffer and 50 µl of 2 X loading buffer (4% w/v SDS, 20% v/v glycerol, 0.002% w/v bromophenol blue, 1 M β-mercaptoethanol, in 1 M Tris [pH 6.8]). After boiling each sample for 3 minutes, samples were electrophoretically separated on an SDS polyacrylamide gel (4% w/v stacking gel and 7.5% w/v resolving gel) according to the method of Laemmli (1970) (Appendix).

Protein was stained with coomassie blue stain for 30 minutes followed by destaining in 50% v/v methanol/10% v/v acetic acid. Each gel was vacuum dried and exposed to X-ray film for 3 days in an X-ray cassette. X-ray film was developed in an automatic film processor.

2.2.3.4 **Immunohistochemistry**

2.2.3.4.1 *Introduction*

The location of proteins are able to be detected with high specificity and sensitivity at the cellular level using the technique of immunohistochemistry. Immunostaining was carried out using polyclonal primary antibodies coupled with biotinylated secondary antibodies in an indirect detection method.

2.2.3.4.2 *Immunohistochemistry Method*

Immunohistochemistry was performed using the avidin-biotin-peroxidase method of Hsu *et al.* (1981). Sections were deparaffinised by passing the slides through three changes of xylene for 10 minutes each. Rehydration was through an alcohol series of decreasing ethanol concentrations (96%, 96%, 90%, 90%, 70%) for 2 minutes each. A well to hold the incubation buffer was formed by applying a ring of wax around each section using a purpose-designed wax pen. Slides were immersed in phosphate-buffered saline (PBS) for 3 minutes. To inhibit endogenous peroxidase activity, sections were then treated with 1% v/v hydrogen peroxide for 10 minutes. Non-specific binding was blocked in 2% v/v normal goat serum diluted in 5% w/v bovine serum albumin (BSA). Each section was incubated with blocking agent for 30 minutes in a humidified chamber equilibrated with water. Blocking agent was removed by rinsing the slides in PBS for 5 minutes. Primary antibody (50 - 100 µl) was applied to each section at the appropriate concentration (Chapter 3). From stock, primary antibody was diluted in a solution of 1% w/v BSA, 0.02% w/v sodium azide in PBS. Sections were incubated for 2 days with primary antibody at 4°C in a humidified chamber equilibrated with water. Slides were then rinsed in PBS for 10 minutes to remove excess primary antibody. Following washing, 50 - 100 µl biotinylated secondary antibody was applied to each section. Either goat anti-rabbit IgG (dilution 1:30) or horse anti-mouse IgG (dilution 1:500) was used as the secondary antibody as appropriate. Secondary antibody was diluted

from stock in 1% w/v BSA, 0.02% w/v sodium azide in PBS. Sections were incubated for 2 hours at room temperature in a humidified chamber equilibrated with water. Excess secondary antibody was then removed by rinsing in PBS for 10 minutes. The avidin-biotin peroxidase complex (50 - 100 µl) diluted 1:30 in 1% w/v BSA, 0.02% w/v sodium azide in PBS was then applied to each section and incubated for 2 hours at room temperature. Slides were rinsed for 10 minutes in PBS. Antibody-bound peroxidase was visualised with DAB. Approximately 200 µl of DAB substrate was applied to each section and the reaction carried out at room temperature for 2 to 5 minutes until the brown immunostain developed. The reaction was immediately stopped by placing slides in PBS for 5 minutes. Sections were lightly counterstained in Carazzi's haematoxylin for 2 minutes and mounted in Eukitt (Appendix).

2.2.3.5 **Western Analysis**

2.2.3.5.1 *Protein Extraction*

Microdissected growth plates from 82 and 140 days gestation (0.4 - 1.3 g) were pulverised to powder in a tissue compactor containing liquid nitrogen.

Protein was extracted with TRIzol® according to a protocol supplied by the manufacturer (Life Technologies, 1995) and based on the method described by Chomczynski and Sacchi (1987). Tissues were processed in TRIzol® as already described for RNA extraction (section 2.2.3.2.2). While the aqueous phase was processed for RNA the lower red phenol-chloroform phase was further processed to yield protein. DNA from the lower red phenol-chloroform phase was removed by precipitation with 96% ethanol (0.3 ml per 1 ml starting volume). Following mixing and incubation at room temperature for 3 minutes, the DNA was pelleted by centrifugation at 2 000 g for 5 minutes at 4°C. Protein was precipitated from the phenol-ethanol supernate with the addition of propan-2-ol (1.5 ml per 1 ml starting volume). Samples were kept at room temperature for 10 minutes and centrifuged at 12 000 g for 10 minutes at 4°C. The protein pellet was then washed three times for 20 minutes each in a 0.3 M guanidine hydrochloride/96% ethanol solution. After the final wash, the pellet was washed in 2 ml 96% ethanol, incubated for 20 minutes at room temperature and centrifuged at 7 500 g for 5

minutes at 4°C. The pellet was dried and then resuspended in 4 ml 1% w/v SDS per 1 g of starting material. Incubation at 50°C assisted resuspension of the pellet. Protein was stored at -20°C until use.

2.2.3.5.2 *Western Immunoblotting*

Proteins were electrophoretically separated on an SDS polyacrylamide gel (4% w/v stacking gel and 15% w/v resolving gel) according to the method of Laemmli (1970) (Appendix). The gel was soaked in protein transfer buffer for 20 minutes before electroblotting proteins onto nitrocellulose membrane. The membrane was pre-wet in distilled water followed by equilibration in protein transfer buffer for 10 minutes. Electroblotting was carried out for 1 hour at 8 v/cm according to manufacturers instructions in an electroblotting apparatus (Appendix). Protein on the membrane was visualised by staining with Ponceau S for five minutes followed by destaining in distilled water (Salinovich and Montelaro, 1986). Immunoblotting was performed essentially as recommended by the membrane manufacturer (Amersham International Plc, 1995). Non-specific binding sites were blocked by immersing the membrane in 4% w/v non-fat dried milk dissolved in Tris-buffered saline (TBS) containing 0.05% v/v Tween-20. Incubation was carried out with agitation for 2 hours at room temperature. The membrane was washed briefly in TBS containing 0.1% w/v BSA. Each membrane received 5 ml of primary antibody diluted to an appropriate concentration recommended by the supplier. IGF-I was diluted 1:1000, while IGF-II was diluted 1:500 from stock, into TBS containing 0.1% w/v BSA. Incubation was carried out overnight at 4°C with agitation. The primary antibody was removed and the membrane washed three times for 15 minutes each in TBS containing 0.1% v/v Tween-20. Goat anti-rabbit IgG secondary antibody labelled with peroxidase was used in an indirect detection method. Secondary antibody was diluted to 1:10 000 in a solution of TBS containing 0.1% w/v BSA. The membrane was covered with 5 ml secondary antibody and incubated at room temperature for 2 hours. Three 15 minute washes of the membrane were carried out in TBS containing 0.1% v/v Tween-20. The membrane was then washed in TBS for 15 minutes. Results were visualised by enhanced chemiluminescence. An ECL detection kit was used according to manufacturers instructions (Amersham International Plc, 1995).

2.2.3.6 Plasma Analysis of Labelled IGF-I

Plasma samples were analysed to determine the clearance of circulating forms of ^{125}I-labelled IGF-I. This was achieved by chromatography at neutral pH on a 1 X 30 cm Superose 12 column fitted to a Fast Protein Liquid Chromatography column. The Superose 12 column was designed for fractionation within the range 1 000 - 300 000 daltons. Separation was based on molecular weight enabling detection of bound and free forms of ^{125}I-IGF-I. Analyses were carried out according to the method described by Hodgkinson *et al.* (1987). Plasma samples were centrifuged at 2 500 g for 30 minutes at 4°C. Each sample (100 µl) was applied to a Superose 12 column, previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.4) (containing sodium azide, 0.01% w/v) and eluted with the same buffer at 0.5 ml/minute. Fractions of 0.5 ml/minute (0.25 ml) were collected and the radioactive content measured in a gamma counter.

2.2.3.7 Tracer Detection

2.2.3.7.1 *Autoradiography*

Sections from *in situ* hybridisation, the receptor binding assay and animals infused with ^{125}I-IGF-I were exposed to liquid emulsion to determine the cellular localisation of labelled tracer (probe or peptide) at the level of light microscopy. Under safelight conditions in a dark room, emulsion was diluted 1:1 v/v (NTB 2 emulsion) or 1:1 w/v (K5 emulsion) from stock. In a tube, 5 ml of milli-Q water was added to 5 ml melted stock NTB 2 emulsion or 5 ml of milli-Q water was displaced up to a 10 ml mark with stock K5 emulsion. The emulsion was melted for 30 minutes at 40°C. Approximately 100 µl/section (depending on section size) was dispensed along the edge of the slide. The emulsion was spread over the section by passing a pasteur pipette along the face of the slide. Each slide was placed upright in a rack and left to dry at room temperature in a lightproof box with desiccant (activated silica gel). After drying, the slides were transferred to a smaller lightproof box and exposed with desiccant for an appropriate period at 4°C (Hahn, 1983). Inclusion of a drying agent was important since humidity increases fading of latent images in emulsion (Haase *et al.*, 1984). The exposure period was determined empirically. As a general rule, if a suitable image was obtained after 24 hours exposure to X-ray film, then exposure to emulsion was for 10 days.

Emulsion background was tested by coating and developing a clean, unused slide and visualising grain density by light microscopy. If the number of grains per field of view at 100 X magnification was less than 100, it was deemed to be an acceptable level (Rogers, 1989). Additional sections that were processed identically except for an absence of labelled tracer, served as controls for background grains, chemography and latent image fading of the autoradiograph as recommended by Rogers (1989).

After an appropriate exposure period slides were equilibrated to room temperature to prevent condensation, and developed (Haase *et al.*, 1984). Under safelight conditions slides were immersed in D-19 developer (18 - 20°C) for 3 minutes followed by 1 minute in 0.2% v/v acetic acid (18 - 20°C), 10 minutes in Ilofix fixer (18 - 20°C) and thorough rinsing in running tap water for 30 minutes in the light. Sections were counterstained as appropriate (Appendix).

2.2.3.7.2 *Measurement of Radioactive Content*

The radioactive content of probes, radioiodinated peptide, plasma and tissue samples from animals infused with labelled IGF-I was determined by measuring a sample in either a liquid scintillation counter (β-emitter) or gamma counter (γ-emitter) set to count at the appropriate part of the spectrum for each radioisotope. ^{35}S, ^{33}P and ^{32}P were measured in a liquid scintillation counter whereas ^{125}I was measured in a gamma counter.

Liquid Scintillation Counter

Between 0.5 - 2 µl of probe was mixed with 0.4 - 1 ml liquid scintillant and measured for 1 minute across the appropriate range for the particular isotope. An average cpm for each sample was obtained.

Gamma Counter

The radioactive content of plasma, radiolabelled ligand or tissue (between 0.5 - 2 ml or 0.5 - 2 g, as appropriate) were measured in the gamma counter for 30 seconds. A measure of cpm for each sample was obtained.

CHAPTER 3

OPTIMISATION AND CONTROL EXPERIMENTS

This Chapter describes experiments that were carried out to optimise the methods used for analysing mRNA and protein in bone. It also details appropriate control experiments that were undertaken to validate results obtained with methods used in this thesis.

3.1 METHOD DEVELOPMENT

3.1.1 Histological Analyses

3.1.1.1 **Immediate Versus Delayed Fixation**

Collecting bones for RNA analyses is a compromise between speed and precision. When there is no urgency to process bones quickly following animal death, dissection of all soft tissue (particularly time consuming in younger animals) and microdissection of individual regions within bone, can be carried out. This enables tissue that will be fixed to be cut to a similar size prior to fixation and frozen tissue to be more accurately sampled prior to freezing (further dissection of bone is extremely difficult once the tissue is frozen). However, upon death endogenous ribonucleases released from lysosomes can degrade RNA. This occurs at a rate dependent on their concentration in a particular tissue (Beintema *et al.*, 1988). Therefore, it was important to determine the time frame required for dissection of sheep bones used in this thesis for RNA analyses.

Walker and McNicol (1992) have shown that mRNA in post mortem samples of intestine, pancreas, cerebellum and pituitary was stable when there was 24 hours between death and fixation. If after their collection, bones could be kept up to one day before being fixed, it would allow more time for dissection of a large number of bones on the same day and reduce the likelihood of skeletal muscle contamination in those methods requiring RNA or protein extraction from whole bone. The stability of mRNA following death was investigated in bone from a late gestation sheep. Epiphyses from two different long bones were collected and processed as described (Chapter 2). For each bone, half was processed immediately while the other half was stored at 4°C for 24 hours and then processed in the usual way. Sections were analysed for polyadenylated RNA (poly A^+ RNA) by *in situ* hybridisation. Levels of poly A^+ RNA were compared between bones fixed 1 hour and bones fixed 1 day following death.

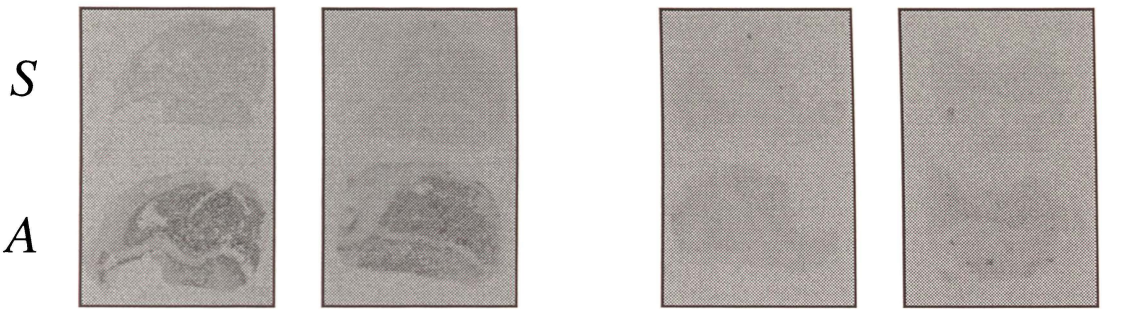
There was a substantial loss of specific hybridisation in the bone samples not fixed until one day following death (Figure 3.1a). As a result of this finding, subsequent tissue collection and dissection was carried out as soon as possible after animal death (usually within 1 hour).

3.1.1.2 **Bone Decalcification**

Analysis of protein or RNA in bone is technically challenging because of its extreme hardness. In order to produce histological sections of bone, modifications to the standard soft tissue processing method need to be made. Prior to processing into paraffin blocks, bones were decalcified to make them soft enough for sectioning. The ability to detect RNA in sections after decalcification has not been well characterised. A few studies have reported successful detection of mRNA by *in situ* hybridisation following decalcification in 20% EDTA (pH 7.4) or a combination of EDTA and HCl (Hirakawa *et al.*, 1994; Tuan, 1993). Therefore several decalcifying agents were tested for their ability to decalcify late gestation bones quickly, whilst maintaining the integrity of RNA. The optimum decalcification agent was further characterised for its effect on RNA and protein when bone was decalcified for different lengths of time.

Figure 3.1 (a, b)

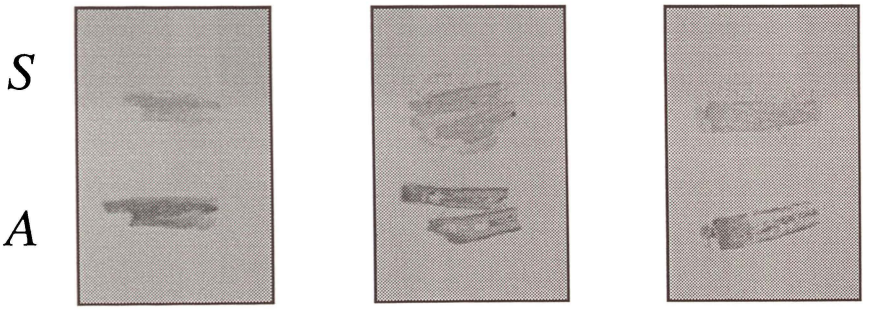
Sections were analysed by *in situ* hybridisation for total mRNA using a sense and antisense poly A^+ probe in adjacent sections.
A represents antisense hybridisation
S represents sense hybridisation
3.1a Autoradiographic images of late gestation epiphyses fixed immediately or 24 hours following animal death. X-ray film exposure was for 25 hours.
3.1b Autoradiographic images of mid gestation diaphyses decalcified using different solutions as indicated. X-ray film exposure was for 1 day.



Different bones from the same animal
Immediate Fixation

Different bones from the same animal
Delayed Fixation

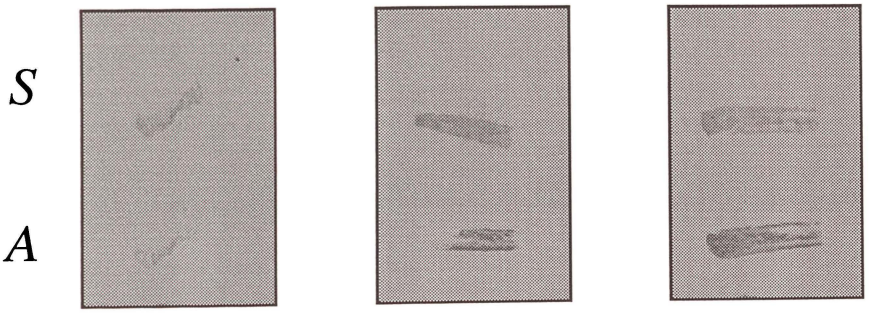
3.1a Time Until Fixation - poly A^+ RNA



5% formic acid

0.2 N HCl

10% acetic acid



commercial decalcifying agent

0.2 M EDTA/ 50mM tris

saturated EDTA

3.1b Decalcifying Agent - poly A^+ RNA

3.1.1.2.1 *Decalcifying Agent*

The diaphyses of a mid gestation sheep were fixed in the standard way and decalcified in either 0.2 N HCl (Becker *et al.*, 1986; Mundlos *et al.*, 1992); commercial decalcifying agent; 5% v/v formic acid (Mullink *et al.*, 1985); 5% v/v trichloroacetic acid (Shi *et al.*, 1992); 10% v/v acetic acid (Mullink *et al.*, 1985); 10% w/v EDTA, pH 7.4; (Alarid *et al.*, 1992); saturated EDTA (Haynes, 1990); or 0.2 M EDTA/50 mM Tris (pH 7.5) (Keene *et al.*, 1991).

After appropriate decalcification (assessed subjectively), bones were processed in the standard way and analysed for poly A^+ RNA by *in situ* hybridisation (Chapter 2). The 10% EDTA solution and the 5% trichloroacetic acid solution were unsuccessful at decalcifying bone and therefore bones receiving this treatment could not be analysed for mRNA. Poly A^+ mRNA was detectable in bone sections decalcified by all the other decalcifying agents (Figure 3.1b). Problems with non-specific probe binding and tissue adherence seen with this and other experiments are discussed later in this Chapter.

Formic acid (5%) was chosen as the decalcification solution for all subsequent bone processing since mRNA could be detected following use, it decalcified bone faster than the other solutions tested, it preserved bone morphology adequately, and acid conditions have previously been shown to be the least destructive on a range of antigens (compared to solutions containing EDTA) (Mullink *et al.*, 1985; Becker *et al.*, 1986).

3.1.1.2.2 *Decalcifying Time*

Since the optimum time for decalcifying larger or older bones was different to the time required for younger or smaller bones, the effect of decalcifying time on mRNA and protein was investigated.

Left and right hind limbs from animals at 50 days gestation (foetal ontogeny study) were collected and fixed in the standard way (Chapter 2). All right hind limbs were decalcified in 5% v/v formic acid at 4°C for 1 day and processed into paraffin blocks for sectioning. Left hind limbs were either decalcified for 1 day, 5 days, 12 days or 19 days in 5% v/v formic acid at 4°C before being processed into paraffin blocks and sectioned. Left and right hind limbs

were analysed for IGF-II mRNA by *in situ* hybridisation. The intensity of hybridisation between matching left and right hind limbs from the same animal were compared (Figure 3.2a). Left and right hind limbs were also analysed by immunohistochemistry. The intensity of immunostaining between matching left and right hind limbs from the same animals were compared (Figure 3.2b).

There was no difference in the relative level and localisation of specific hybridisation or immunostaining between left and right hind limbs regardless of how long the bones had been exposed to formic acid (Figure 3.2). Bones were therefore decalcified for a period of up to 20 days, for all subsequent bone collections.

To further test this result, sections from foetal sheep liver that had been treated with formic acid for 1 day were compared to sections from the same tissue that had not been treated. The acid treatment had no effect on the ability to detect IGF-II mRNA by *in situ* hybridisation or IGF-I and IGF-II by immunohistochemistry (data not shown).

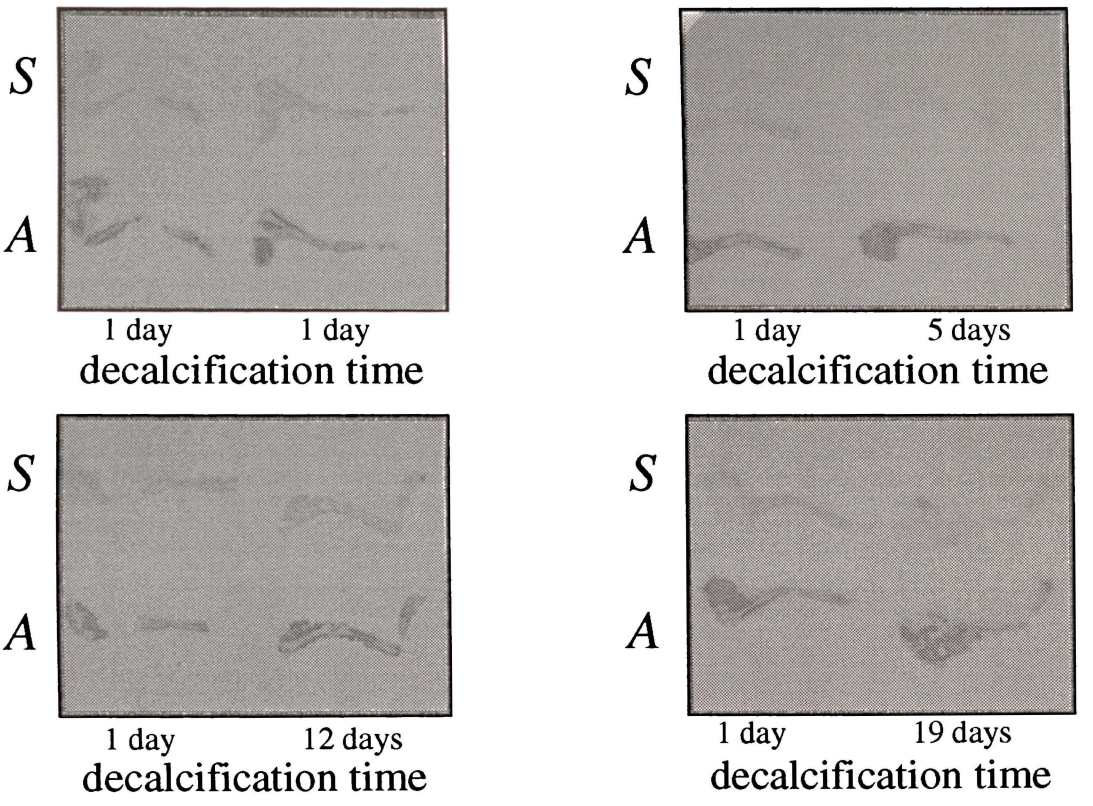
3.1.1.3 Section Adherence

In agreement with Aaron and Carter (1987), this study found that bone sections were difficult to retain on the slide during histological analyses, compared to soft tissue sections. Sections of bone repeatedly lifted off the slides during *in situ* hybridisation and immunohistochemistry. Initially, sections were mounted on gelatin-chrome alum coated slides (Gall and Pardue, 1969). Although this was effective for soft tissues, bone sections failed to adhere during harsh procedures, particularly the high temperatures required for *in situ* hybridisation. Purpose-designed Superfrost plus slides were tested for bone sections, as these had been shown to be superior to other forms of slide treatment (Wilcox, 1993). However, sections continued to lift off the slide despite extensive baking at 45°C prior to use.

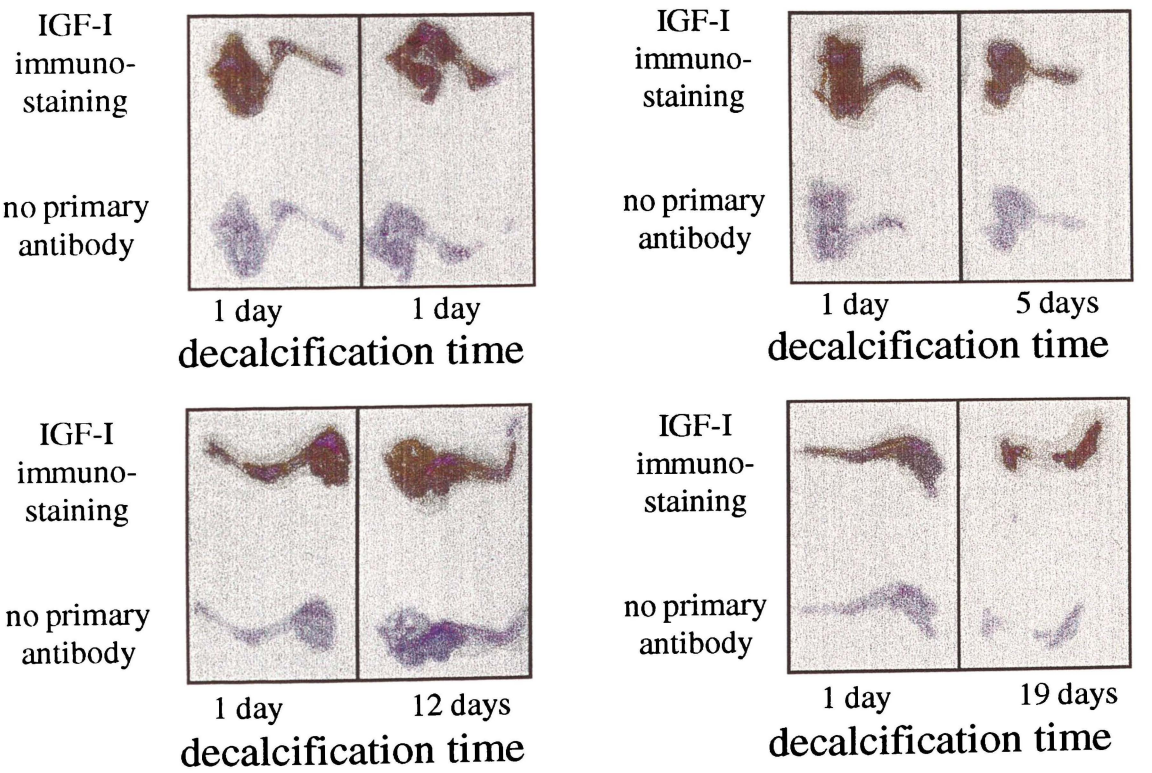
Successful adherence of bone sections required slides to be coated with 3-aminopropyltriethoxysilane, prior to section mounting (Appendix). This treatment is believed to result in covalent bonds forming between 3-aminopropyltriethoxysilane and the aldehyde or

Figure 3.2 (a, b)

The effect of decalcification on the ability to detect mRNA and protein in hind limbs from 50 days gestation. All right limbs were decalcified for 1 day while the corresponding left limbs were decalcified for either 1 day, 5 days, 12 days or 19 days. Right and left hind limbs from the same animal were compared.
3.2a Autoradiographic images obtained from sections analysed by *in situ* hybridisation for IGF-II mRNA. X-ray film exposure was for 1 day.
A represents antisense hybridisation
S represents sense hybridisation in adjacent sections
3.2b Immunoreactive staining (brown colour) in sections analysed by immunohistochemistry for IGF-I. The adjacent section was a negative control where IGF-I Ab was omitted.



3.2a *In Situ* Hybridisation - IGF-II mRNA



3.2b Immunohistochemistry - IGF-I

ketone groups on the section (Rentrop *et al.*, 1986). Slides prepared in this way were successfully used for *in situ* hybridisation and immunohistochemistry analyses.

3.1.2 *In Situ* Hybridisation

Extensive optimisation of *in situ* hybridisation conditions and control experiments to assess hybridisation specificity for probes used in the current studies, have previously been carried out (Molenaar, 1995; Hodges, 1992). Some additional experiments were undertaken to optimise conditions for bone tissue.

3.1.2.1 **Hybridisation Temperature**

The temperature of hybridisation is a major factor that affects the stability of cRNA-mRNA hybrids and hence the specificity of the reaction (Angerer *et al.*, 1987). In general, it is recommended that *in situ* hybridisation be carried out under low stringency conditions at an optimum temperature of Tm minus 25°C (Angerer *et al.*, 1987). For most probes used in the present study, the theoretical hybridisation temperature was calculated to be within the range 35°C to 50°C (Appendix). Hybridisation in tissues other than bone has previously been carried out at 52°C with probes used in this thesis.

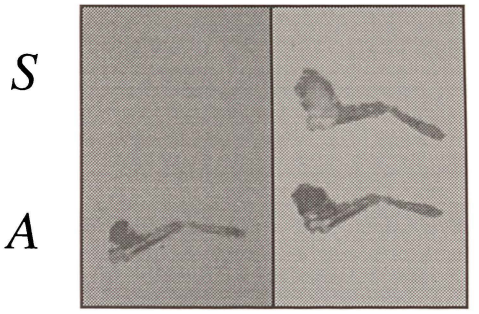
However, most probes gave a high background with the sense strand when hybridised at 52°C in bone sections (Figure 3.3a). The temperature was therefore raised to 58°C for all probes (except poly A^+, which was optimised at 35°C). The higher temperature improved the stringency of hybridisation and resulted in lower background (Figure 3.3a).

3.1.2.2 **Postnatal Animals - Technical Difficulties**

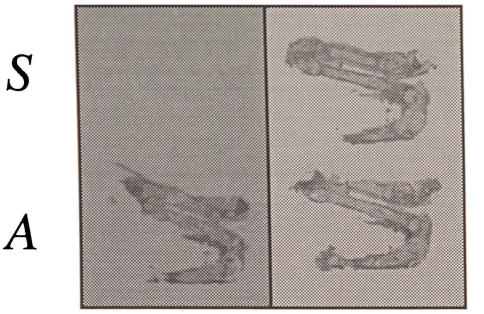
Attempts to process postnatal sheep bones for histological analyses by the method employed for foetal sheep bones were largely unsuccessful. Adequate decalcification in 5% formic acid could not be achieved for the first set of postnatal bones collected. They were microdissected with difficulty and processed into blocks after 30 days of decalcification. However, sections shattered when cut, contained holes and folds and adhered poorly to slides during *in situ*

Figure 3.3 (a, b)

3.3a Bone sections from 50 and 62 days gestation were analysed by *in situ* hybridisation for IGF-II mRNA using sense and antisense probes in adjacent sections. Hybridisation was carried out at either 52°C or 58°C.
X-ray film exposure was for 21 hours (hybridisation at 52°C) or 8 hours (hybridisation at 58°C).
3.3b Photomicrographs of bone sections from 140 days gestation analysed by *in situ* hybridisation for IGF-I and IGF-II mRNA using sense and antisense probes in adjacent cryosections.
Slides were exposed to emulsion for 70 days.
Each photomicrograph represents the proliferative zone of the growth plate.
pr, proliferative chondrocyte
Bar = 100 μm
A represents antisense hybridisation
S represents sense hybridisation

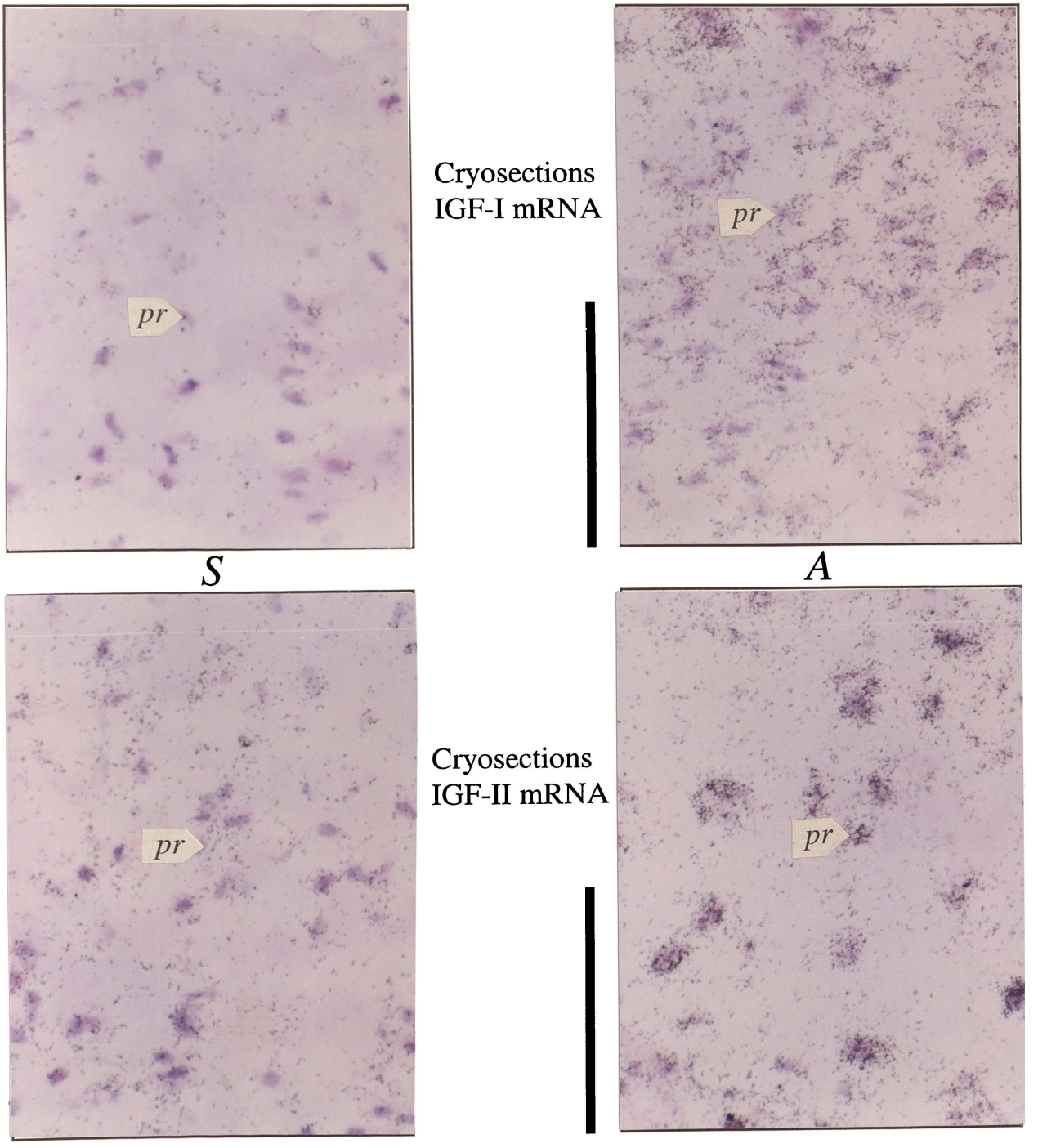


Adjacent hind limbs 50 days gestation



Adjacent hind limbs 62 days gestation

3.3a Effects of Hybridisation Temperature



3.3b Cryosections analysed by *in situ* hybridisation

hybridisation. Investigations in postnatal bone from other species such as rat has also been limited by technical difficulties associated with the calcification of bone (Wang *et al.*, 1995).

Bones were collected from additional animals and processed in a slightly different way to improve decalcification. Bones were sawn into smaller pieces prior to fixation. They were decalcified for either 3 weeks (bones collected from 2 day or 2 week lambs) or 7 weeks (bones collected from 6 month or 18 month old sheep) in several changes of formic acid at 4°C. The acid solution was mixed frequently during decalcification.

Although the softness of bone was improved, sectioning was still very difficult and tissue adherence during *in situ* hybridisation remained a problem, particularly in the older bones. In addition, problems were encountered with background binding of IGF-I and IGF-II sense probes on these sections. Despite repeated experiments to optimise *in situ* hybridisation conditions, background could not be lowered. Local production of IGF-I and IGF-II mRNA in postnatal sheep bones could therefore not be determined for the postnatal period.

3.1.3 Extraction Procedures

3.1.3.1 **RNA Extraction Procedures**

A rapid RNA extraction method utilising a commercial product called TRIzol® and based on the procedure described by Chomczynski and Sacchi (1987) was successful for tissues such as muscle and liver but not for skeletal tissue (Chapter 2, RNA extraction method 1). The extensive extracellular matrix of skeletal tissues poses a problem to successful isolation of RNA. Due to their high buoyant density, proteoglycans in the matrix contaminate RNA extracted by most conventional extraction procedures (Nemeth *et al.*, 1989).

A kit which was designed for rapid and pure isolation of mRNA and developed for use in tissues such as cartilage was trialled for isolation of mRNA from skeletal tissue (Biotek Ltd, 1993). In this method, mRNA in crude tissue extract binds with oligo T sequences covalently linked to the surface of Dynabeads. However, this method resulted in low yields and partially degraded RNA and was therefore abandoned.

The most successful procedure for extraction of RNA from skeletal tissue combined steps from several methods. This method was subsequently used for all total RNA extraction from skeletal tissue (Chapter 2, RNA extraction method 2). It involves homogenisation in guanidinium thiocyanate (Chomczynski and Sacchi, 1987), low speed centrifugation and filtering (Nemeth *et al.*, 1989) and ultracentrifugation in cesium trifluoroacetate following acid phenol extraction (Zarlenga and Gamble, 1987; Chomczynski and Sacchi, 1987).

3.1.3.2 Protein Extraction Procedures

Protein was isolated from dissected growth plates utilising either acid (method described below) or TRIzol® extraction (Chapter 2).

Protein was extracted under acid conditions following the method of D'Ercole and Underwood (1987). Acid conditions are required to dissociate IGFs from their binding proteins. Tissue was pulverised to powder in a tissue compactor containing liquid nitrogen. On ice, powdered tissue was added to 5 volumes/g ice-cold 1 M acetic acid. After vigorous shaking samples were incubated on ice for 2 hours. Samples were then centrifuged at 1 000 g for 10 minutes at 4°C. The supernatant was neutralised with 5M NaOH to give a pH between 6 and 8. The supernatant was stored at -20°C until use.

Due to distortion during electrophoresis, protein samples isolated by this method were unable to be utilised for Western analysis. Extraction using TRIzol® was subsequently utilised (Chapter 2).

3.2 CONTROL EXPERIMENTS

3.2.1 *In Situ* Hybridisation

3.2.1.1 Positive Controls

3.2.1.1.1 *Cryosections (calcified) versus Paraffin Sections (decalcified)*

Most studies report results of mRNA localisation in calcified bone sections. Bone is either sectioned from calcified frozen bone using a specialist microtome, or processed into plastic blocks which do not require prior decalcification for sectioning (Dodds *et al.*, 1993; Ikeda *et al.*, 1992; Hill and Elde, 1990b). Both of these methods are technically difficult and require specialist equipment. The current studies utilised decalcified paraffin embedded bone since it is easier to section and provide better tissue morphology. Fewer studies have analysed mRNA in bone processed by this method, so it was necessary to compare mRNA localisation in bone sections from calcified cryosections and decalcified paraffin sections.

Bone cryosections (10 μm) from six foetal ages (3 animals per age) were prepared as described (foetal ontogeny study, Chapter 2). They were analysed by *in situ* hybridisation for IGF-I and IGF-II mRNA. Results were compared to those obtained with paraffin bone sections from the same animals. Tissue adherence and histology were poor in the cryosections. Where comparisons were possible, the pattern of IGF-I and IGF-II mRNA localisation was similar between decalcified paraffin and calcified frozen sections (Figure 3.3b and Chapter 5). It was concluded that decalcified paraffin sections were acceptable for mRNA analyses.

3.2.1.1.2 *rRNA*

In order to test the integrity and hybridisability of RNA and to optimise *in situ* hybridisation, each section was hybridised with a probe coding for the predominant RNA species found in cells - 18S and 28S ribosomal RNA (rRNA). This positive control was used to support results where cells or regions of tissue were found to be negative with probes used to localise components of the IGF system. Ribosomal RNA has previously been evaluated by *in situ*

hybridisation and found to be a suitable tool for determining levels of hybridisable RNAs in tissue sections (Yoshii *et al.*, 1995).

All sections of bone and soft tissue were analysed with a human ribosomal RNA probe by *in situ* hybridisation. A typical result is shown in Figure 3.4 for bone sections from representative animals of the foetal ontogeny study (Chapter 2). Ribosomal RNA was consistently high in all sections where it was measured, which is not surprising since ribosomal RNA forms a significant part of total RNA in every cell (Yoshii *et al.*, 1995). Only a short exposure of sections to X-ray film was required to obtain results (compared to exposure periods required for mRNA species). Relative levels of probe binding did not change over development, between bones from a particular animal or between animals within an age group (Figure 3.4). At the cellular level, rRNA could be detected in all cells.

It was concluded from these results that rRNA was appropriate to be used as a positive control for the tissues investigated in the current study. Where appropriate the cellular localisation of ribosomal RNA is presented in other Chapters of this thesis alongside the results of mRNA localisation.

3.2.1.2 **Negative Controls**

3.2.1.2.1 *α lactalbumin*

The specificity of hybridisation in bone sections was investigated using a probe for mRNA known to be absent in bone. A probe for α lactalbumin mRNA was used since α lactalbumin is a milk whey protein specific to the mammary gland (Hurley and Schuler, 1987). The α lactalbumin probe was similar in length and specific activity to other probes used in the current studies.

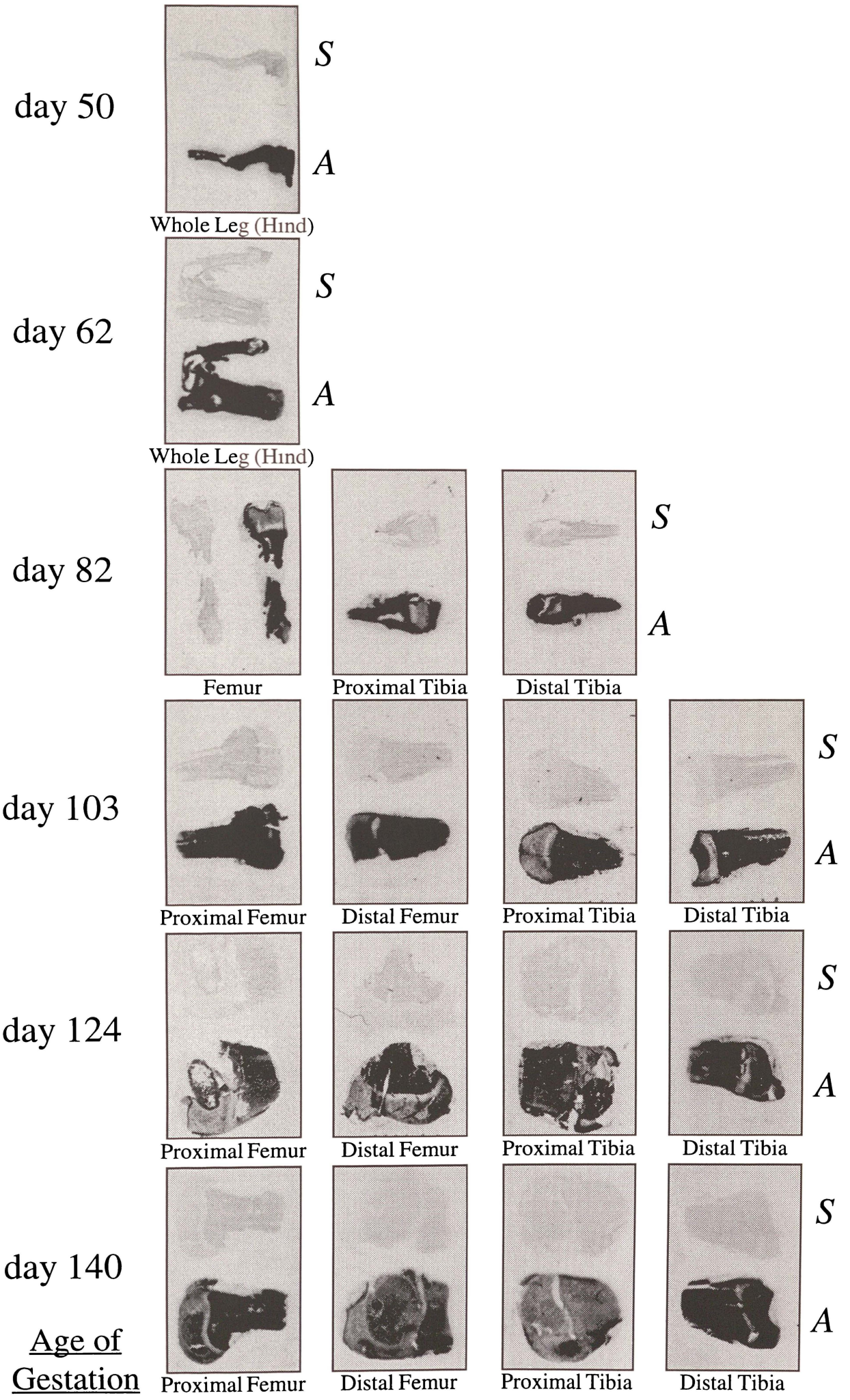
In situ hybridisation with the α lactalbumin probe was carried out on bone sections from animals in the foetal ontogeny study (Chapter 2). Additionally, sheep mammary gland was included to confirm probe integrity.

Figure 3.4
POSITIVE CONTROL FOR *IN SITU* HYBRIDISATION

Autoradiographic images of bone sections from the foetal ontogeny study (50-140 days gestation) probed with either an antisense or sense ribosomal RNA probe in adjacent sections. Bones from one representative animal at each age are presented. X-ray film exposure was for 21 hours.

A represents antisense hybridisation

S represents sense hybridisation in adjacent sections


day 50
S
A
Whole Leg (Hind)
day 62
S
A
Whole Leg (Hind)
day 82
S
A
Femur
Proximal Tibia
Distal Tibia
day 103
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 124
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 140
S
A
Age of Gestation
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia

There was an absence of specific hybridisation in all bone sections (Figure 3.5). This was evident on X-ray film even after a long exposure, and at the cellular level. While bone sections were negative for α lactalbumin mRNA, mammary gland tissue displayed a strong signal on X-ray film, confirming the integrity of the probe. mRNA was localised to areas of active alveoli, in agreement with that which has been reported previously (Molenaar *et al.*, 1992).

It was concluded from these results that an absence of particular mRNA species in bone sections is accurately assessed as being absent by *in situ* hybridisation.

3.2.1.2.2 *Sense Hybridisation- Control for Non-Specific Hybridisation*

Oppositely orientated promoters either side of template DNA enabled RNA probes to be transcribed that were antisense or sense with respect to target mRNA. Since the sequence of sense probes is identical to target mRNA, binding of these probes reflects non-specific binding in tissue sections. They have the same specific activity, concentration and fragment length as their antisense counterpart making them suitable negative controls for *in situ* hybridisation (Angerer *et al.*, 1987).

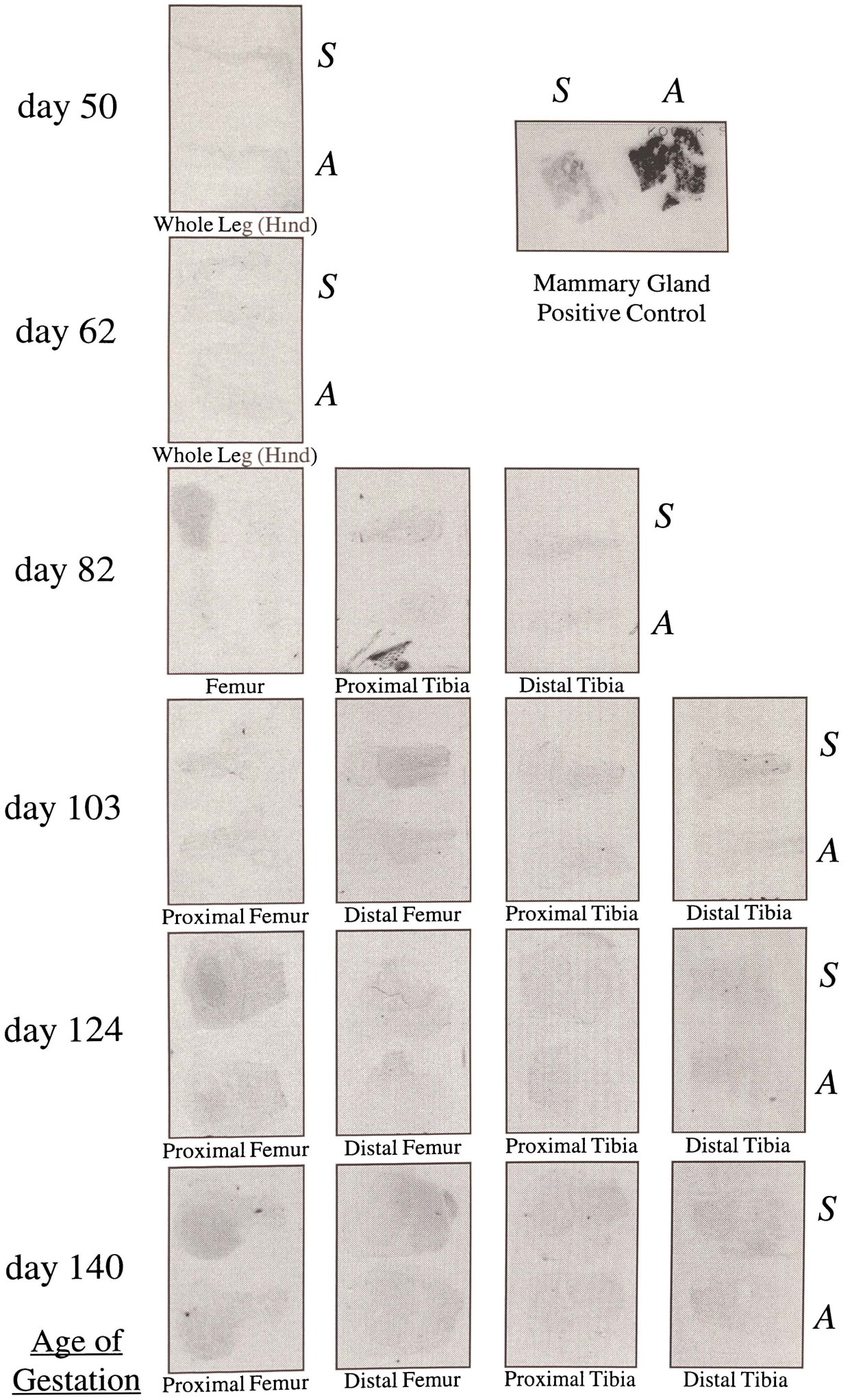
For every *in situ* hybridisation experiment undertaken, two adjacent sections were placed on each slide. One section was probed with an antisense probe (signal) while the other section was probed with a sense probe (background). Specific (cRNA-mRNA) formation is therefore the difference in signal observed with the antisense probe (specific cRNA-mRNA formation and non-specific probe binding to unidentified cellular constituents or other nucleic acids with weak homology) and the signal observed with the sense probe (non-specific probe binding to unidentified cellular constituents or other nucleic acids with weak homology).

Depending on exposure period, probe, and the particular experiment, variable but low sense probe binding was observed (see results for each *in situ* hybridisation experiment - Chapters 3, 4, and 5). Within a particular *in situ* hybridisation experiment, sense probe binding was found to be similar for all sections. When antisense probe binding was higher than sense probe binding, it was concluded that there was specific mRNA in the tissue for the particular mRNA

Figure 3.5
NEGATIVE CONTROL FOR *IN SITU* HYBRIDISATION

Autoradiographic images of bone sections from the foetal ontogeny study (50-140 days gestation) or a sheep mammary gland section probed with either an antisense or sense. α lactalbumin probe in adjacent sections. Bones from one representative animal at each age are presented. X-ray film exposure was for 2 days.

A represents antisense hybridisation
S represents sense hybridisation in adjacent sections


day 50
S
A
Whole Leg (Hind)
S
A
Mammary Gland
Positive Control
day 62
S
A
Whole Leg (Hind)
day 82
S
A
Femur
Proximal Tibia
Distal Tibia
day 103
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 124
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 140
S
A
Age of Gestation
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia

species that was being analysed. The bigger the difference (within a particular experiment), the higher the abundance of mRNA. Where there was no specific binding (antisense and sense probe binding gave the same signal intensity), it was concluded that the particular mRNA was absent or below the level of sensitivity of the *in situ* hybridisation technique.

3.2.2 Northern Analysis

Northern hybridisation was used in this thesis to determine the presence or absence of particular mRNA species from the IGF system and to confirm probe specificity. Results from Northern hybridisation assisted in the validation of results obtained by *in situ* hybridisation. For a number of reasons, quantification of particular mRNA species between two different RNA samples was not undertaken (discussed in Chapter 5). This is in part due to the difficulty in comparing RNA derived from mixed tissues, the variability in the ratio of 18S and 28S ribosomal fractions found within a sample and the necessity for complex evaluation and experimentation for valid quantification of RNA (Spanakis and Brouty-Boylé, 1994).

3.2.2.1 **Skeletal Muscle Contamination**

It was not possible to dissect out individual bones from the youngest animals (gestational ages 50 and 62) or to completely remove all skeletal muscle from the outside of older bones collected for the foetal ontogeny study. Therefore, bone microdissected from frozen tissue for RNA extraction was likely to contain not only bone, but a variable amount of skeletal muscle. Since foetal sheep skeletal muscle is known to contain IGF-I and IGF-II mRNA (Hodges, 1992), it was important to ascertain which RNA samples contained skeletal muscle, to assist in the interpretation of IGF-I and IGF-II mRNA specific in bone.

Northern hybridisation using a probe specific to skeletal muscle (α skeletal actin) was carried out with RNA isolated from foetal sheep skeletal muscle (positive control), adult sheep liver (negative control) and RNA samples isolated from the growth plate or diaphysis of one animal at 50, 62, 82, 103, 124 and 140 days gestation (foetal ontogeny study). Results indicate that this probe was specific for α skeletal actin. Skeletal muscle but not liver RNA contained α

skeletal actin mRNA (1.5 kb mRNA) (Figure 3.6a). 1.5 kb is consistent with the reported size for α skeletal actin mRNA (Davey and Wildeman, 1995).

Results show that RNA samples extracted from microdissected growth plates were not contaminated with skeletal muscle RNA since they did not contain α skeletal actin mRNA (Figure 3.6a). In contrast, RNA samples extracted from tissue at 50 and 62 days gestation contained α skeletal actin mRNA suggesting the presence of skeletal muscle RNA in these samples (Figure 3.6a). RNA samples extracted from the diaphysis of bone, were also found to contain a variable amount of α skeletal actin mRNA.

The presence of skeletal muscle and the integrity of the α skeletal actin probe were confirmed by *in situ* hybridisation in limbs taken from the younger animals (Figure 3.6b). Variable amounts of skeletal muscle surrounded bone at 50 and 62 days gestation. When compared to the localisation of IGF-II mRNA in adjacent sections, α skeletal actin mRNA was found exclusively in skeletal muscle, while IGF-II mRNA was found in bone, cartilage and skeletal muscle.

It was not possible to correct for the amount of skeletal muscle RNA in these samples, since the variability in α skeletal actin mRNA levels during foetal sheep development are not well characterised. A difference in the relative level of α skeletal actin mRNA was found between skeletal muscle from the foetal and postnatal periods (Figure 3.6b).

These results indicate that gene expression cannot be credited exclusively to bone in RNA samples from 50 and 62 days gestation or the diaphysis of older animals. However gene expression does appear to be specific to bone tissue in all growth plate samples.

3.2.2.2 **rRNA**

Ribosomal RNA was used as an indicator of sample integrity, to confirm the presence of RNA in the absence of hybridisable mRNA and to equalise the loading of RNA between lanes on a

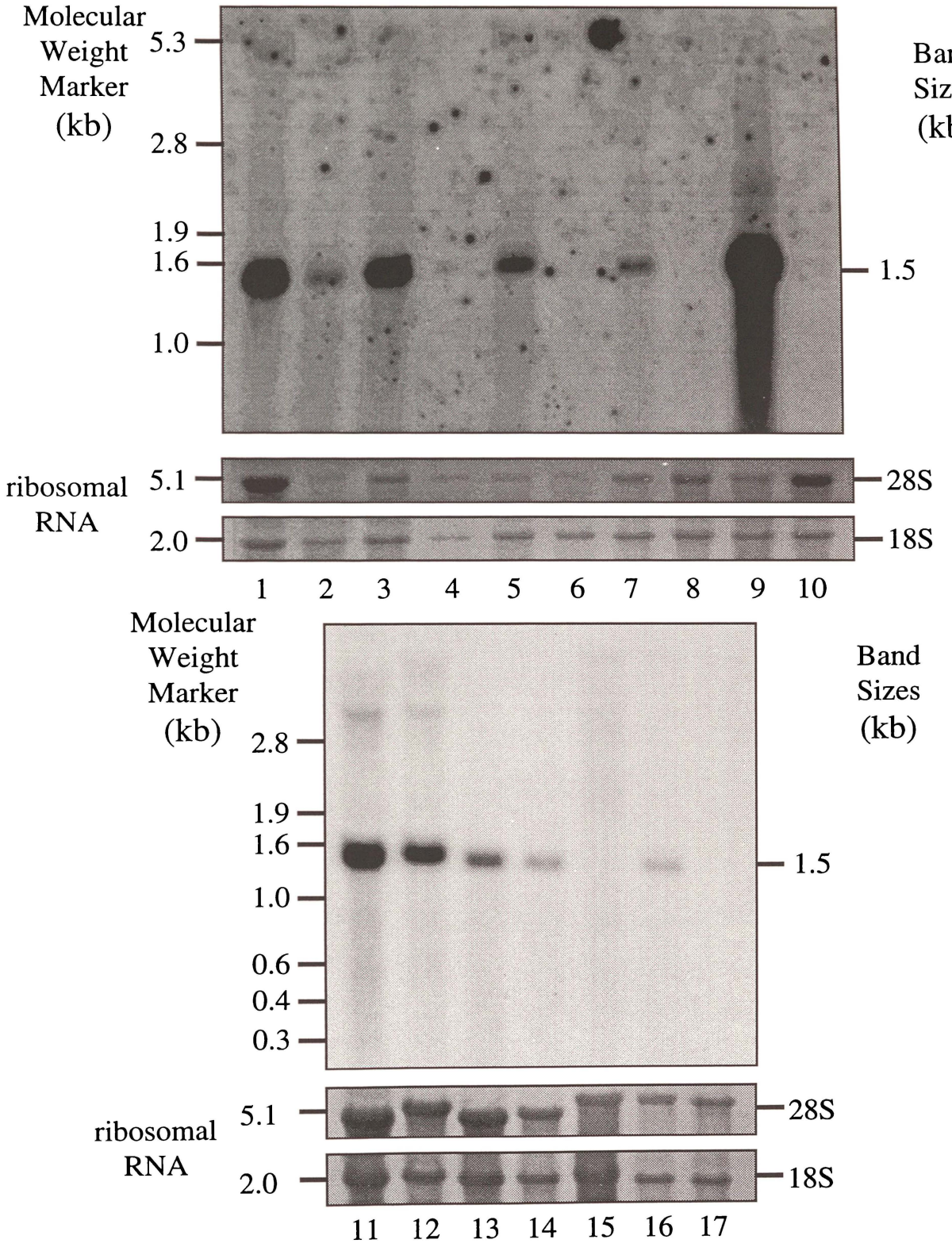
Figure 3.6 (a, b)

3.6a Identification of which RNA samples extracted from microdissected bone were contaminated with skeletal muscle. Total RNA samples (20 μg) were hybridised with an α -skeletal muscle actin probe, specific for skeletal muscle mRNA.
Lower panel shows hybridisation with a ribosomal RNA probe.
Exposure to X-ray film was for 7 days (lanes 1-10) or 1 day (lanes 11-17)

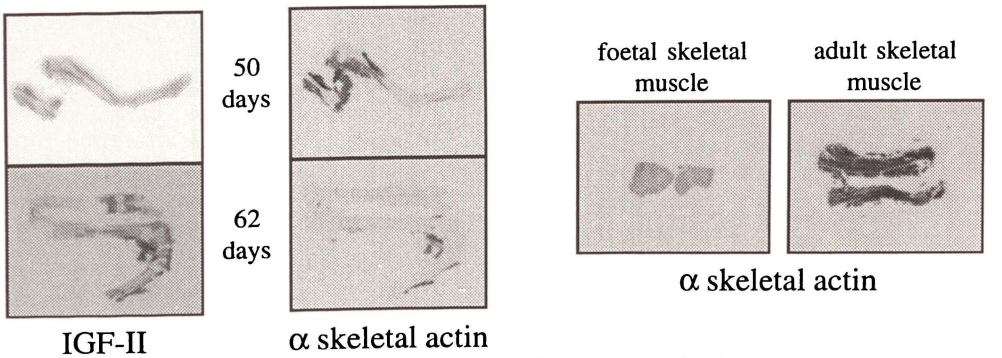
RNA loaded in each lane was as follows:

1 50 days gestation (fore limb)
2 62 days gestation (fore limb)
3 82 days gestation, humerus (diaphysis)
4 82 days gestation, humerus (growth plate)
5 103 days gestation, humerus (diaphysis)
6 103 days gestation, humerus (growth plate)
7 124 days gestation, humerus (diaphysis)
8 124 days gestation, humerus (growth plate)
9 140 days gestation, humerus (diaphysis)
10 140 days gestation, humerus (growth plate)
11 foetal skeletal muscle (20 μg)
12 foetal skeletal muscle (10 μg)
13 postnatal mouse bone (hind limb)
14 postnatal mouse bone (fore limb)
15 adult sheep liver
16 124 days gestation, humerus (diaphysis)
17 124 days gestation, humerus (growth plate)

3.6b Analysis of α -skeletal muscle actin mRNA in bone from 50 days gestation, 62 days gestation, foetal sheep skeletal muscle and adult sheep skeletal muscle by *in situ* hybridisation. Exposure to X-ray film was for 4 days. Comparison to IGF-II mRNA in adjacent sections. All sections were probed with an antisense probe.



3.6a α skeletal actin mRNA in RNA from Bone



3.6b α skeletal actin mRNA in bone and skeletal muscle

gel. Ribosomal RNA has been used extensively for these purposes previously (for example, Duhl *et al.*, 1992; Masters *et al.*, 1992).

It was decided not to use rRNA as a reference for the quantification of specific mRNA species since it has been shown by Spanakis (1993) that analysing mRNA levels in this way can be misleading. This type of analysis relies on the assumption that ribosomal RNA levels are constant between cells and tissues under different physiological conditions. In this study, it was found that the ratio of 28S to 18S ribosomal RNA was different between samples. This could be the result of 28S or 18S ribosomal RNA being selectively removed during RNA isolation in a variable way or normal variation in the ratio of 18S to 28S between tissues.

3.2.3 Immunohistochemistry

The specificity of immunostaining detected by immunohistochemistry needs to be confirmed by a number of experiments (Naish *et al.*, 1989). False positive immunostaining can be the result of non-specific sticking and/or specific cross-reactions (Harlow and Lane, 1988). Control experiments including titration of the primary antibody (Ab), leaving out the primary and/or secondary Abs, replacing the primary Ab with non-immune serum raised in the same species as the primary Ab, using an Ab with a well-characterised pattern of localisation and pre-adsorption of the primary Ab with excess peptide (see Chapter 3 for discussion of this control), were carried out on bone sections from the foetal ontogeny study.

3.2.3.1 **Antibody Titration**

The appropriate concentration of primary Ab was determined empirically. A series of dilutions for IGF-I, IGF-II, BP-2, BP-3, BP-4 and BP-5 Abs were tested to determine the lowest dilution of Ab that could be used to observe immunostaining. For the IGF-I Ab, dilutions of 1:1500, 1:2000, 1:2500 and 1:3000 were tested. The level of immunostaining at these concentrations was similar (Figure 3.7), therefore a second experiment investigated even lower dilutions of the IGF-I Ab (1:5000 and 1:10000). Although immunostaining was still observed at these lower dilutions, the intensity of immunostaining at 1:10000 was slightly

Figure 3.7
PRIMARY ANTIBODY TITRATION

Immunostaining (brown colour) in sections analysed by immunohistochemistry using IGF-I, IGF-II, IGF BP-2, -3, -4 and -5 Abs at various dilutions (as indicated).
The optimum dilution used for subsequent experiments is denoted with an asterix (*).
Adjacent bone sections are from animals at 50 days gestation (IGF-I and IGF-II Abs) or 124 days gestation (IGF BP-2, -3, -4 and -5 Abs).



1:1500

1:2000*

IGF-I Ab



1:2500

1:3000

IGF-I Ab



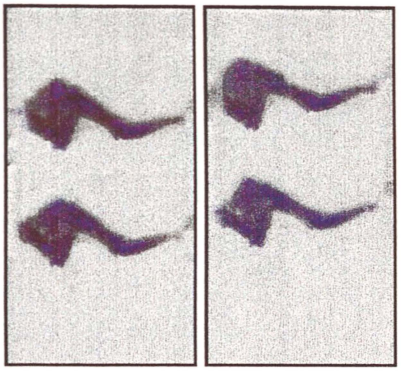
1:5000

1:10000

IGF-I Ab

1:200

1:250*



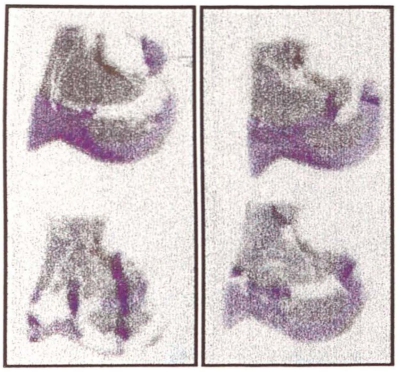
1:500

1:750

IGF-II Ab

1:250*

1:750



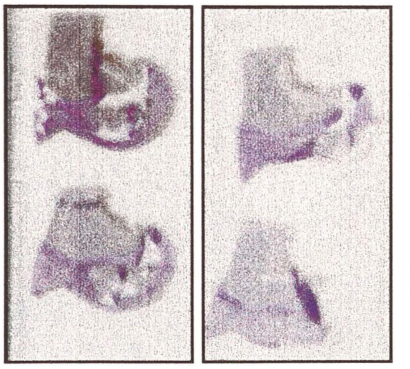
1:500

1:1000

IGF BP-2 Ab

1:100*

1:750



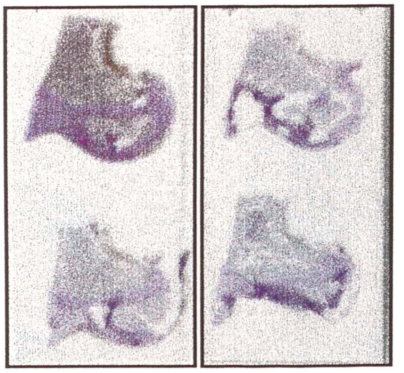
1:500

1:1000

IGF BP-3 Ab

1:100*

1:750



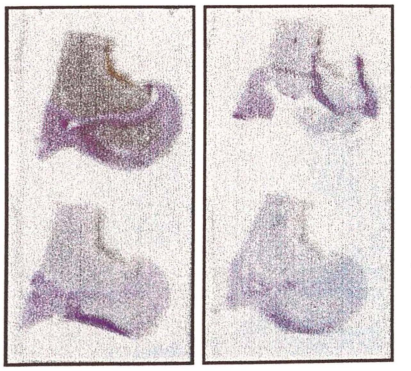
1:500

1:1000

IGF BP-4 Ab

1:100*

1:750



1:500

1:1000

IGF BP-5 Ab

lower than that at 1:5000 (Figure 3.7). All subsequent analyses using the IGF-I Ab were carried out at a dilution of 1:2000, since a loss of staining in some cells was observed at dilutions lower than this (data not shown).

For other Abs used in this thesis, a more obvious fall in immunostaining was observed as the Ab concentration was lowered. The IGF-II Ab was tested at dilutions of 1:200, 1:250, 1:500 and 1:750. Immunostaining decreased dramatically between dilutions of 1:250 and 1:750 (Figure 3.7). All subsequent experiments used the IGF-II Ab at 1:250. The BP-2 Ab was tested at dilutions of 1:250, 1:500, 1:750 and 1:1000 while BP-3, BP-4 and BP-5 Abs were tested at dilutions of 1:100, 1:500, 1:750 and 1:1000. Based on the resulting immunostaining (Figure 3.7), BP-2 Ab was subsequently used at a dilution of 1:250 while BP-3, BP-4 and BP-5 Abs were used at a dilution of 1:100.

3.2.3.2 No 1° and/or No 2° Antibody

When the primary Ab or the secondary Ab or both the primary and secondary Abs were replaced with 1% w/v BSA; 0.02% w/v sodium azide, immunostaining was abolished. This was evident at the tissue level (Figure 3.8) and at the cellular level. Where appropriate, these controls are presented alongside the data in other Chapters.

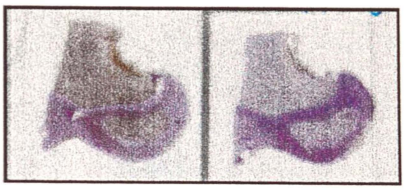
3.2.3.3 Non-Immune Serum Control

Primary Ab was replaced with non-immune rabbit serum at a dilution similar to that used for the primary Ab. Since Abs were used at various dilutions (section 3.3.3.1), dilutions of 1:200, 1:250 and 1:2000 were tested in separate experiments. The level of immunostaining was compared to that of primary Abs (for example, BP-5 or IGF-I) used at their optimum dilution (previous section). Results were variable. On one occasion staining was evident when non-immune serum was diluted at 1:250 but not at 1:2000 (Figure 3.8). However, the intensity of staining observed at 1:250 compared to that of the IGF-I Ab at a 1:1500 dilution was lower (Figure 3.8). In a separate experiment, very weak staining was observed when non-immune serum was diluted at 1:200 compared to BP-5 Ab at a dilution of 1:100 (Figure 3.8).

Figure 3.8 (a, b)
NEGATIVE CONTROLS FOR IMMUNOHISTOCHEMISTRY

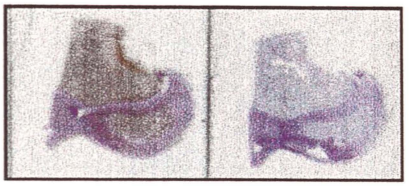
Negative controls for immunohistochemistry carried out on sections from the foetal ontogeny study (50 and 124 days gestation).
3.8a The effect of no primary Ab *or* no secondary Ab *or* no primary Ab and no secondary Ab on immunostaining (brown colour). An adjacent bone section displaying BP-4 immunostaining is represented for comparison. Bone sections are from 124 days gestation.
3.8b The effect of replacing the primary Ab with various dilutions of non-immune serum (1:200; 1:250; 1:2000). This experiment was carried out on two separate occasions in bone sections from 50 and 124 days gestation. An adjacent bone section displaying IGF-I or BP-5 immunostaining (brown colour) is represented for comparison.

POSITIVE NEGATIVE



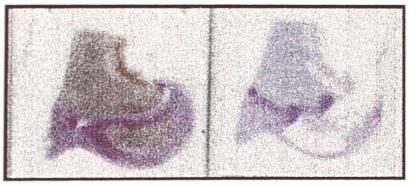
BP-4 Ab — no primary Ab, normal secondary Ab

POSITIVE NEGATIVE



BP-4 Ab — BP-4 primary Ab, no secondary Ab

POSITIVE NEGATIVE



BP-4 Ab — no primary Ab, no secondary Ab

3.8a Negative Control: No 1° and/or No 2 ° Antibody

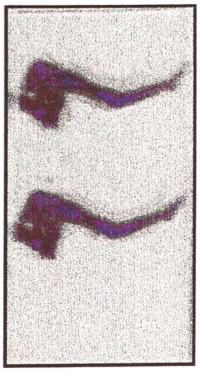
NEGATIVE



non-immune serum 1:250

non-immune serum 1:2000

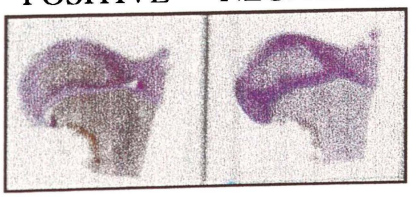
POSITIVE



IGF-I Ab 1:1500

IGF-I Ab 1:2000

POSITIVE NEGATIVE



BP-5 Ab 1:100 — non-immune serum 1:200

3.8b Negative Control: Non-Immune Serum

At the cellular level, weak staining with non-immune serum was largely localised to skeletal muscle, bone cavities and occasionally in the reserve cartilage and hypertrophic zones. All other areas of the growth plate were negative, regardless of the concentration of non-immune serum used.

This weak non-specific staining could be due to a high concentration of protein in the particular non-immune serum used. Naish *et al.* (1989) recommends equalising the protein concentration in non-immune serum to that of the primary Ab for comparisons to determine specific immunostaining. A high concentration of protein in non-immune serum can result in the formation of aggregates or oligomers in non-immune serum or the binding of Abs in non-immune serum to proteins in the tissue which results in positive staining. Where appropriate, the non-immune serum control is presented alongside data.

3.2.3.4 Distribution of Previously Characterised Antibody

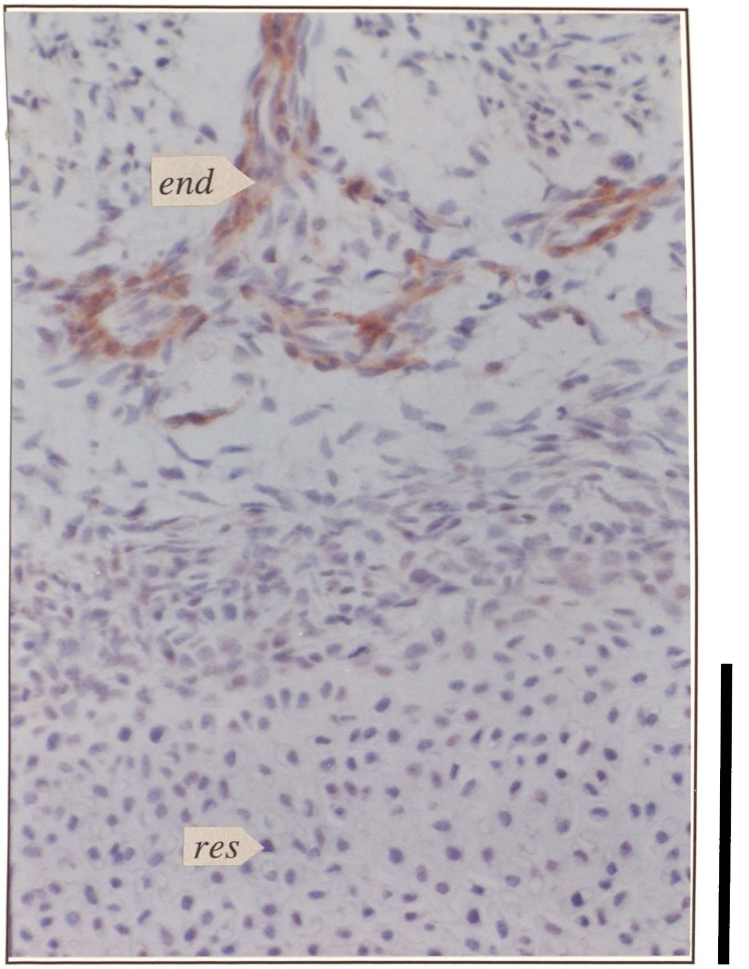
An Ab directed against an antigen with a known cellular distribution was used to determine the specificity of immunohistochemistry in bone sections. The Ab used for this purpose was specific for an endothelial glycoprotein. As expected immunostaining was localised exclusively to blood vessels (Figure 3.9). It was concluded from this result that the broad distribution of immunostaining observed with Abs against members of the IGF system was unlikely to be due to indiscriminate and non-specific binding of primary Abs with these tissues.

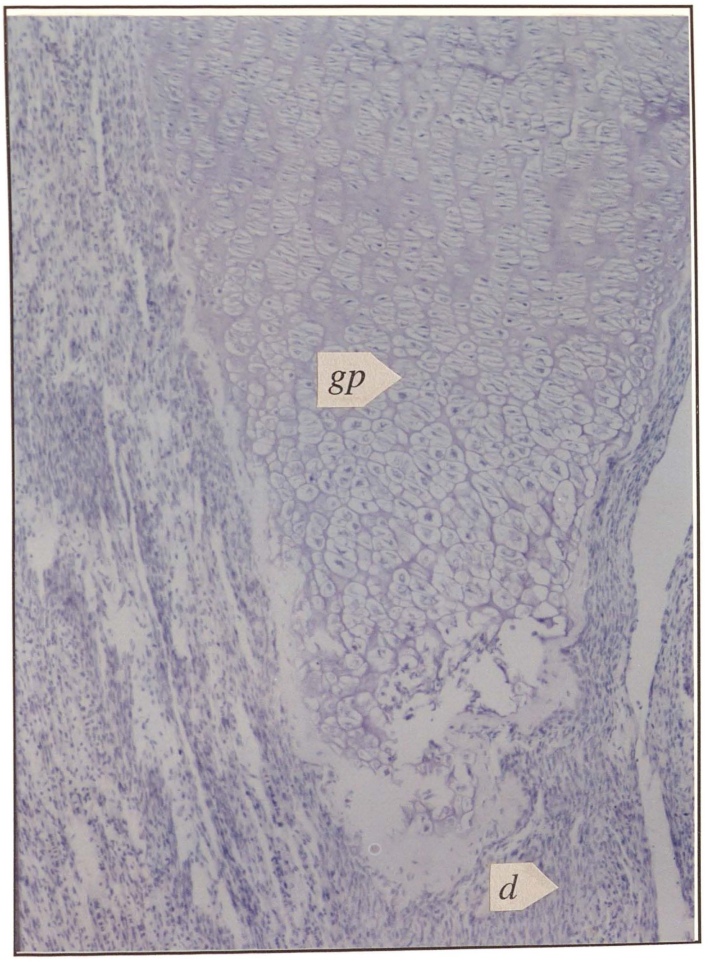
3.2.3.5 Pre-adsorption of Primary Antibody

Another negative control often used to differentiate specific from non-specific staining, involves affinity absorption of the primary Ab with antigen prior to its use (Tolszczuk and Pelletier, 1988; Bloch *et al.*, 1983). This was able to be carried out for the IGF-I and IGF-II Abs, since sufficient quantities of IGF-I and IGF-II peptide were available. In contrast, pre-adsorption of IGF BP Abs was not investigated due to the difficulty in obtaining sufficient quantities of pure protein for all of the IGF BPs. However, a recent study has reported negligible staining when IGF BP-3 and -4 Abs, obtained from the same source as those used

Figure 3.9
NEGATIVE CONTROL FOR IMMUNOHISTOCHEMISTRY

Results obtained with an Ab directed against endothelial glycoprotein. This antigen has a known and well-defined distribution pattern that is unrelated to the areas important to the current study.
Bone sections from the foetal ontogeny study (50 days gestation).
Bone in the diaphysis (*d*), cartilage growth plate (*gp*)and reserve chondrocyte (*res*) were negative. Immunostaining was confined to the endothelial cells of blood vessels (*end*).
Bar = 100 μm


end
res


gp
d

in the present study, were pre-adsorbed with IGF BP-3 and IGF BP-4 protein, suggesting that these Abs are specific for IGF BP-3 and IGF BP-4, respectively (Braulke *et al.*, 1996).

Despite using a range of peptide concentrations (10 ng/ml to 12.5 µg/ml), neither the pre-adsorption of IGF-I Ab with IGF-I peptide or IGF-II Ab with IGF-II peptide, was successful at reducing the level of staining observed with these Abs. This is surprising, given that other studies report success in blocking IGF-I immunostaining with IGF-I peptide at approximately 0.1 µM (8 ng/ml) (Lazowski *et al.*, 1994; Hansson *et al.*, 1988). It is possible that in the present study, the concentration of IGF Ab used was too high to enable it to be completely blocked by IGF. While immunostaining for IGF-I was still observed at a dilution of 1:10 000 with the IGF-I Ab, all pre-adsorption experiments for the IGF-I Ab were carried out at a dilution of 1:2000. Alternatively, there may have been a problem with the integrity of the IGF-I and IGF-II peptides used to pre-adsorb the IGF Abs, that would reduce their ability to bind to the IGF Abs.

It is unlikely that significant cross-reactivity of the IGF-I Ab with IGF-II peptide or IGF-II Ab with IGF-I peptide was occurring, since negligible cross-reactivity was observed on an immunoblot containing pure IGF-I and IGF-II peptide at concentrations ranging from 0.1 to 200 ng peptide. Furthermore, IGF-II peptide was as equally unsuccessful at pre-adsorbing IGF-I Ab as was found for IGF-I peptide, and IGF-I was unsuccessful at pre-adsorbing IGF-II Ab.

CHAPTER 4

LOCALISATION OF IGF-I AND IGF-II, IGF BINDING SITES, AND IGF BINDING PROTEINS -2, -3, -4 AND -5

4.1 INTRODUCTION

IGF-I and IGF-II are major factors involved in bone growth and development (see Introduction). It is believed that this is the net result of widespread effects on cell replication and the production of proteins associated with differentiation. The effects of IGF-I and IGF-II are not only similar but are apparently ubiquitous in cartilage and bone cells. However, most of the actions of IGF-I and IGF-II have been studied in cell culture. Isolated cells may not behave as they do *in vivo* since cell growth *in vivo* is a more tightly controlled and coordinated process involving regulation by multiple growth factors and interactions between cells of the same and different regions.

The major components of the IGF system involved in generating a biological response are the Type I and Type II IGF receptors, IGF-I and IGF-II, and the IGF binding proteins. It is important to determine the availability of each component in particular cells throughout development to characterise potential spatial and temporal patterns of IGF action. In separate studies, IGFs, IGF receptors and IGF BPs have been identified in bone, but their localisation has only been partially characterised. Only one study has investigated the co-localisation of components from the IGF system across bone development (Wang *et al.*, 1995).

The aim of the present study was to identify the potential role that IGFs play in the development and growth of foetal sheep long bones by investigating components of the IGF system. This was achieved by characterising the localisation of IGF receptors, IGF-I, IGF-II and IGF BPs -2, -3, -4 and -5.

Binding sites for IGF-I and IGF-II were analysed in growth plates from three different foetal ages. Further studies were carried out to determine whether this binding was to Type I and Type II IGF receptors and/or IGF BPs. The presence of Type I and Type II IGF receptors was investigated in microsomal membranes isolated from the growth plate. The location of IGF BPs -2, -3, -4 and -5 was determined in bones during foetal development and the potential for local production of IGF BPs -2, -3, -4 and -5 was also investigated by *in situ* hybridisation.

4.2 METHODS

4.2.1 IGF-I and IGF-II in Foetal Bone Development

To investigate the presence of IGF-I and IGF-II in bone, sections were analysed by immunohistochemistry and extracted protein was analysed by Western blotting, as described in Chapter 2. For IGF-II, both a polyclonal (all foetal ages) and monoclonal (50 days gestation) IGF-II antibody were used and the results compared.

Bone sections representing the proximal and distal femur and tibia from one animal at each age of the foetal ontogeny study were analysed. To simplify discussion, the presence of immunostaining for IGF-I and IGF-II will subsequently be referred to as the presence of IGF-I staining, IGF-II staining, IGF-I or IGF-II. Sections were visually assessed and results summarised for each bone and age. Relative immunostaining was an arbitrary measure of the relative intensity of immunostaining coupled with the number of immunoreactive cells. It was assessed on a five point scale from absent to high.

Appropriate controls for immunohistochemistry (described in Chapter 3) were carried out. It was concluded that immunostaining was specific for IGF-I or IGF-II omitting the primary and/or secondary antibody resulted in an absence of immunostaining. Replacing the primary antibody with an equivalent dilution of non-immune serum resulted in negligible immunostaining (see Chapter 3 for discussion of these controls).

Protein was extracted from microdissected growth plates from an animal at 82 days gestation and an animal at 140 days gestation. These samples were analysed by Western blotting for IGF-I and IGF-II. Protein isolated from other foetal sheep tissues previously reported to contain IGF-I and IGF-II in other species (Hill and De Sousa, 1990a; Ralphs *et al.*, 1990; Hill *et al.*, 1989), were also analysed.

4.2.2 Binding Sites for IGF-I and IGF-II

Calcified sections of bone (proximal humerus) were analysed for the localisation of IGF-I and IGF-II binding sites. Due to technical difficulties associated with sectioning calcified bone, analyses were restricted to the growth plate from a limited number of animals. Sections representing three animals from three ages of the foetal ontogeny study (62, 103 and 140 days gestation) were chosen for comparison. Cryosections were cut and mounted onto slides and assayed for IGF-I and IGF-II binding as described in Chapter 2.

For the characterisation of IGF-I binding, four adjacent sections were incubated with either ^{125}I-IGF-I (total binding), ^{125}I-IGF-I and IGF-I (1 μg/ml) (non-specific binding), ^{125}I-IGF-I and IGF-II (1 μg/ml) (related peptide) or ^{125}I-IGF-I and insulin (10 ng/ml). Similarly, the characterisation of IGF-II binding was assessed by incubating four adjacent sections with either ^{125}I-IGF-II (total binding), ^{125}I-IGF-II and IGF-II (1 μg/ml) (non-specific binding), ^{125}I-IGF-II and IGF-I (1 μg/ml) (related peptide) or ^{125}I-IGF-II and insulin (10 ng/ml).

Specific binding was visually assessed at the cellular level as the difference between total binding and non-specific binding. Binding in the presence of competing ligand indicated the

level of cross-reactivity between binding sites for ^{125}I-IGF and the ligand. If there was no displacement of binding with either of the competing ligands (related IGF or insulin), it was considered that the binding site had a high affinity for ^{125}I-IGF. Any displacement of ^{125}I-IGF with competing ligand(s) was an indication of the relative affinity of the binding site(s) for ^{125}I-IGF, the related ligand and insulin.

4.2.2.1 Characterisation of Membrane Binding Sites

To determine whether IGF-I and IGF-II were binding to the Type I and Type II IGF receptors, microsomal membranes were isolated from a microdissected growth plate from the proximal humerus of a 140 day gestation animal (Chapter 2). ^{125}I-IGF-I and ^{125}I-IGF-II were affinity cross-linked to protein in the presence (non-specific binding) and absence (total binding) of competing IGF-I (1 μg/ml) or IGF-II (1 μg/ml), respectively.

The size of the affinity cross-linked products was determined by electrophoresis on an SDS polyacrylamide gel (Chapter 2). Specific binding was assessed as the difference between total binding and non-specific binding.

4.2.2.2 Localisation of IGF Binding Proteins -2, -3, -4 and -5

To determine whether the binding sites found for IGF-I and IGF-II in the growth plate could be the result of binding to IGF BPs, growth plates were analysed by immunohistochemistry for the most common IGF BPs found in bone (IGF BP-2, -3, -4 and-5). Additionally, the co-localisation of IGF BPs -2, -3, -4 and -5 to regions of bone found to be positive for IGF-I and IGF-II was assessed.

Bone sections representing the proximal and distal femur and tibia from one animal at each age of the foetal ontogeny study were analysed for IGF BP-2, -3, -4 and-5 by immunohistochemistry, as described in Chapter 2. The presence of immunostaining for each IGF BP will subsequently be described as IGF BP (positive) staining or IGF BP. Sections were visually assessed and results summarised for each bone at all ages and for each IGF BP. Relative immunostaining was an arbitrary measure of the relative intensity of immunostaining

coupled with the number of immunoreactive cells. It was assessed on a five point scale from absent to high.

The appropriate controls for immunostaining (as described in Chapter 3 and above for IGF-I and IGF-II detection) were carried out. Immunostaining was concluded to be specific for each IGF BP, as omitting the primary antibody and/or the secondary antibody resulted in no staining. Additionally, replacing the primary antibody with an equivalent dilution of non-immune serum resulted in negligible staining.

4.2.3 Local Production of IGF BP-2, -4 and -5 mRNA

The local production of IGF BP-2, -4 and -5 mRNA in foetal bone development was investigated by Northern hybridisation and *in situ* hybridisation. RNA extracted from the diaphysis and growth plate of one animal at each age of the foetal ontogeny study were analysed. These samples were the same ones used for IGF-I, IGF-II and α skeletal actin mRNA analyses (Chapters 3 and 5). Membranes containing these samples were stripped and rehybridised with probes for IGF BP-2, -4 and -5, as described in Chapter 2.

To confirm the results found by Northern hybridisation, the presence of IGF BP-2, -4 and -5 mRNA was investigated at the cellular level by *in situ* hybridisation. Sections of femur (proximal and distal), and tibia (proximal and distal) from one animal at each age of the foetal ontogeny study were analysed. To confirm probe integrity, sections from various rat tissues known to express IGF BP-2, -4 and -5 were included.

4.3 RESULTS

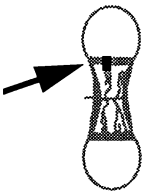
4.3.1 IGF-I and IGF-II in Foetal Bone Development

It was concluded that staining for IGF-I and IGF-II was specific. Omitting either IGF and/or secondary antibody resulted in an absence of immunostaining (Figure 4.1). Similarly,

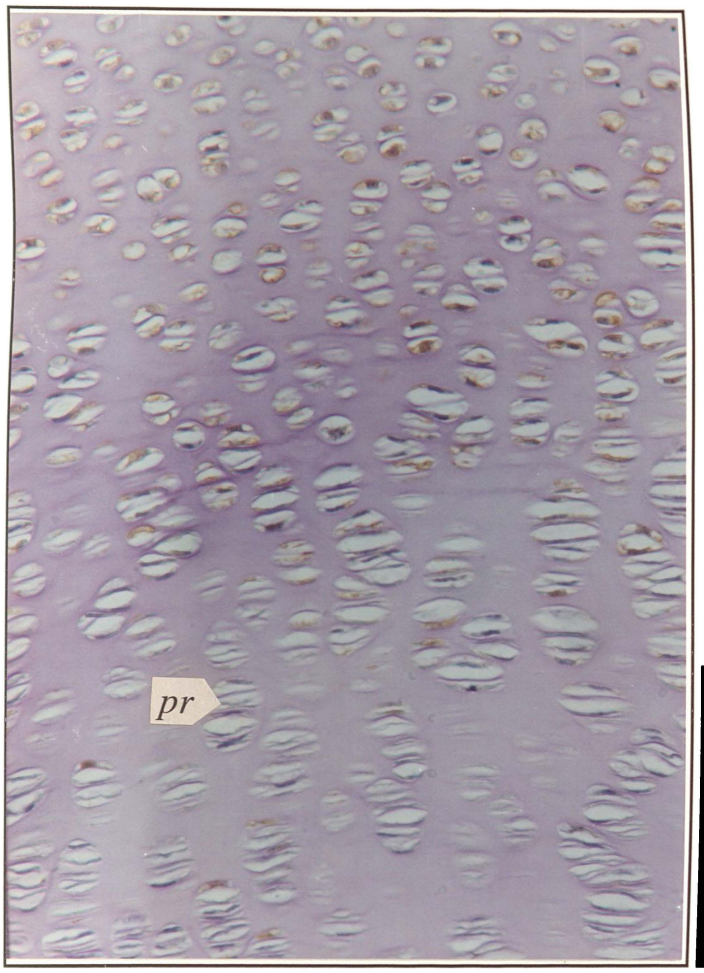
Figure 4.1 (a, b)
CONTROLS FOR IMMUNOHISTOCHEMISTRY

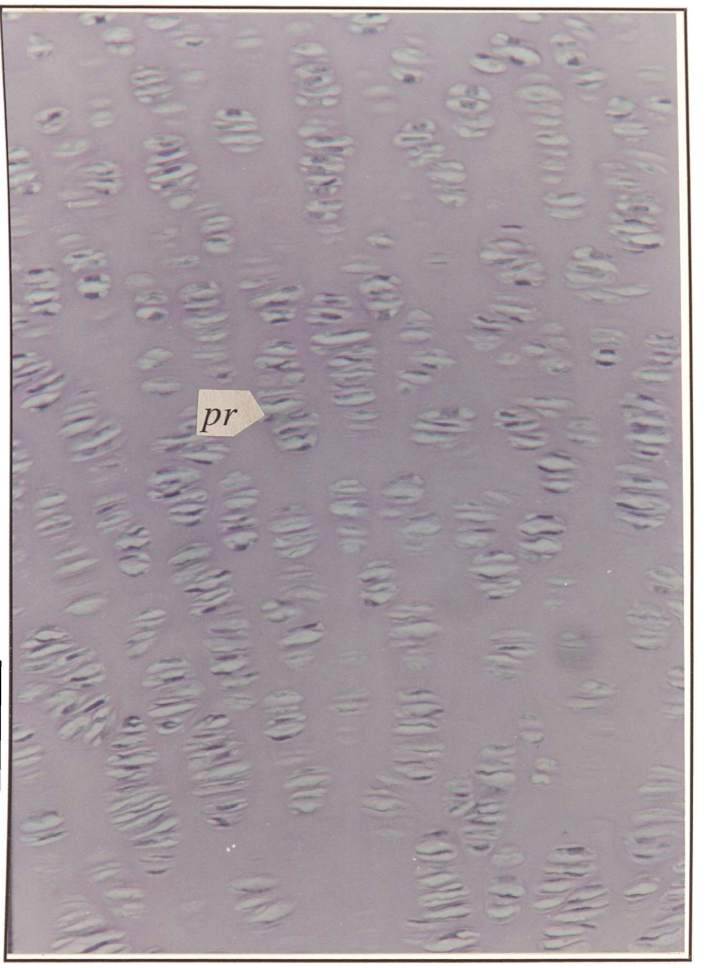
Bone sections from the proximal femur of a 124 day gestation animal from the foetal ontogeny study. Sections were either incubated with non-immune serum instead of primary antibody (a) or 1% w/v BSA, 0.02% w/v sodium azide in PBS instead of primary antibody (b).

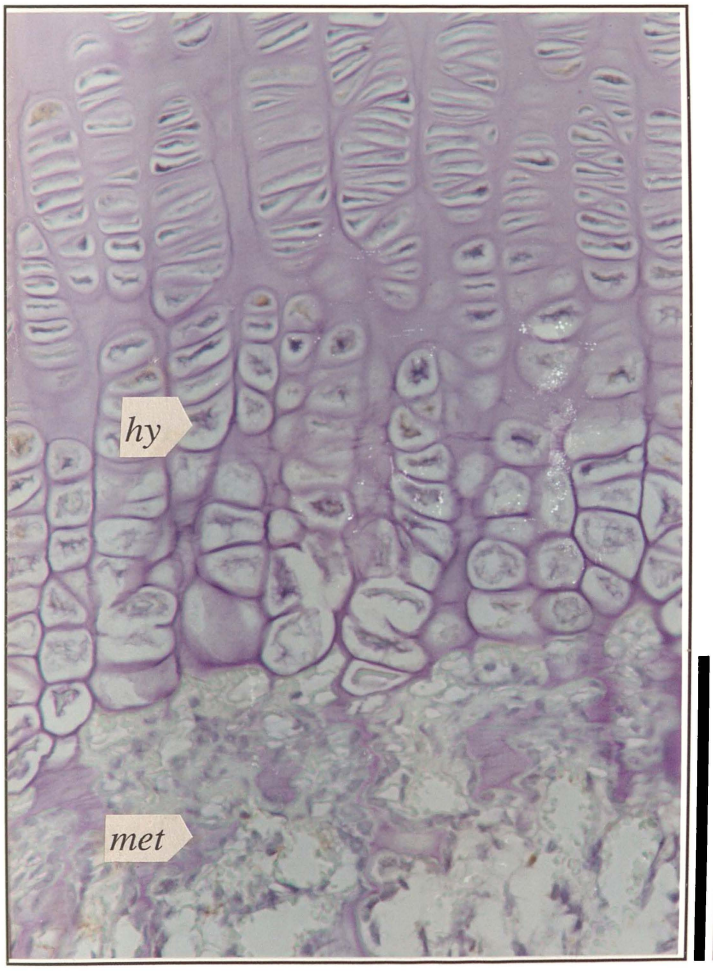
Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)

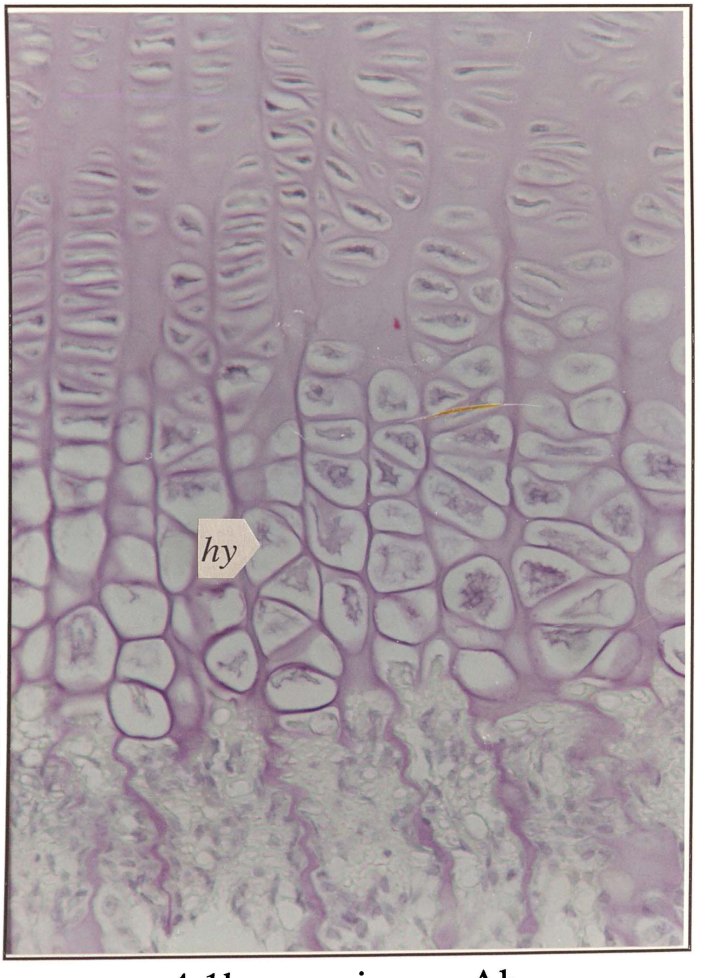


pr, proliferative chondrocyte; *hy*, hypertrophic chondrocyte; *met*, metaphysis
Vertical Bar = 100 μm









4.1a non-immune serum

4.1b no primary Ab

replacing the primary antibody with an equivalent dilution of non-immune serum resulted in negligible immunostaining (Figure 4.1). Particular cells positive for IGF-I, IGF-II, IGF BPs and IGF binding sites were identified by their histological appearance and location within bone. The characteristics of these cells have been described in detail in Chapter 1.

4.3.1.1 Distribution of IGF-I and IGF-II

IGF-I and IGF-II were similarly located in all areas of bone and at all stages of foetal development (Tables 4.1 and 4.2). A similar result for IGF-II localisation was obtained with both a polyclonal and monoclonal IGF-II antibody (Table 4.2). Overall, IGF-I and IGF-II were widely distributed in areas of cartilage and bone (Tables 4.1 and 4.2). In cartilage, IGF-I and IGF-II were restricted largely to chondrocytes from the reserve cartilage and hypertrophic zones of the growth plate throughout development. Osteoblasts stained the most intensely for IGF-I and IGF-II in bone. However other cells from the bone cell lineage and the bone matrix were also stained.

4.3.1.1.1 *Chondrogenesis*

Staining was absent in the cartilage matrix in all bone sections regardless of age (Figures 4.2 and 4.3). Within cells, staining was localised to either the cytoplasm or nucleus in some cells or to both the nucleus and the cytoplasm in other cells.

Staining for IGF-I and IGF-II in osteogenic cells residing in the perichondrium was similar at all ages, while staining in the reserve cartilage zone varied with age (Figures 4.2 and 4.3). In the reserve cartilage zone only a few chondrocytes were stained for IGF-I and IGF-II at the younger ages (50, 62, 82 and 103 days gestation) whereas at the older ages (124 and 140 days gestation) the number of positively stained cells increased (Figures 4.2 and 4.3).

In almost all of the younger bones, there was an absence of IGF-I and IGF-II in the proliferative zone. As development proceeded and this zone became narrower, more chondrocytes within proliferating columns stained positively for IGF-I and IGF-II (Figures 4.2 and 4.3). Once chondrocytes began to hypertrophy, the number of stained cells increased. For

IGF-I	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	++	++	++	++	n.d.	n.d.
reserve zone (chondrocytes)	+	++	+	++	+++	+++
proliferative zone (chondrocytes)	–	–	–	–	+++	++
hypertrophic zone (chondrocytes)	+++	+++	+++	+++	++	+++
Cartilage Matrix	–	–	–	–	–	–
periosteum (osteogenic cells)	++	++	++	++	++	+++
bone cavity (osteogenic cells)	n.d.	+	+	++	+	+
osteoblasts	++++	++++	++++	++++	++++	++++
osteocytes	n.d.	+++	+++	+++	+++	+++
Bone Matrix	++	++	++	++	++	++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	++++	++++
muscle fibres	++++	++++	++++	n.d.	n.d.	n.d.

Table 4.1

Summary of the localisation and relative abundance of immunoreactive IGF-I in different cells and regions of bone. Bone sections from one animal per age from the foetal ontogeny study were analysed.
Specific immunostaining was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where immunostaining was not determined; (–) indicates an absence of specific immunostaining

IGF-II	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation	50 days gestation *
perichondrium (chondrogenic cells)	++	++	+	–	n.d.	n.d.	+++
reserve zone (chondrocytes)	++	++	+	++	+++	+++	–
proliferative zone (chondrocytes)	–	+	–	–	++	+	–
hypertrophic zone (chondrocytes)	+++	+	++	++	+	++	+
Cartilage Matrix	–	–	–	–	–	–	–
periosteum (osteogenic cells)	++	++	++	++	++	+++	+++
bone cavity (osteogenic cells)	n.d.	–	++	++	+	+	+
osteoblasts	++++	++++	++++	++++	++++	++++	++++
osteocytes	n.d.	+++	+++	+++	+++	+++	+++
Bone Matrix	++	++	++	++	++	++	++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	++++	++++	++++	n.d.
muscle fibres	++++	++++	++++	n.d.	n.d.	++++	++++

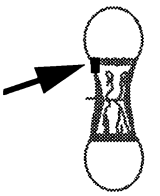
Table 4.2

Summary of the localisation and relative abundance of immunoreactive IGF-II in different cells and regions of bone. Bone sections from one animal per age from the foetal ontogeny study were analysed.
Specific immunostaining was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where immunostaining was not determined; (–) indicates an absence of specific immunostaining
* indicates results of immunohistochemistry using a monoclonal antibody to detect IGF-II.

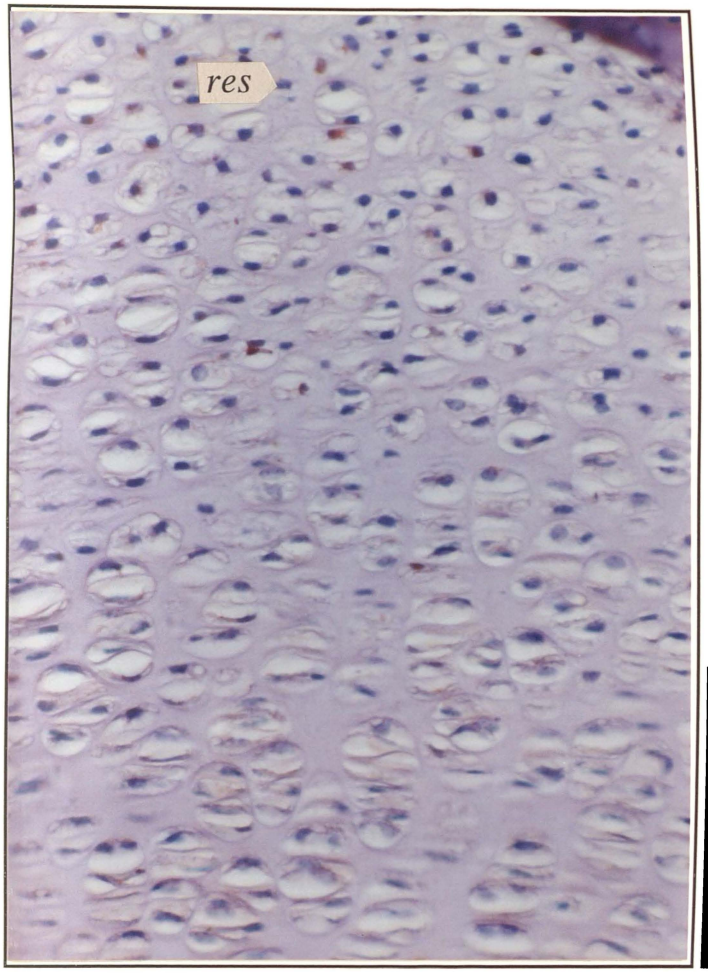
Figure 4.2 (a, b)
LOCALISATION OF IGF-I IN THE GROWTH PLATE

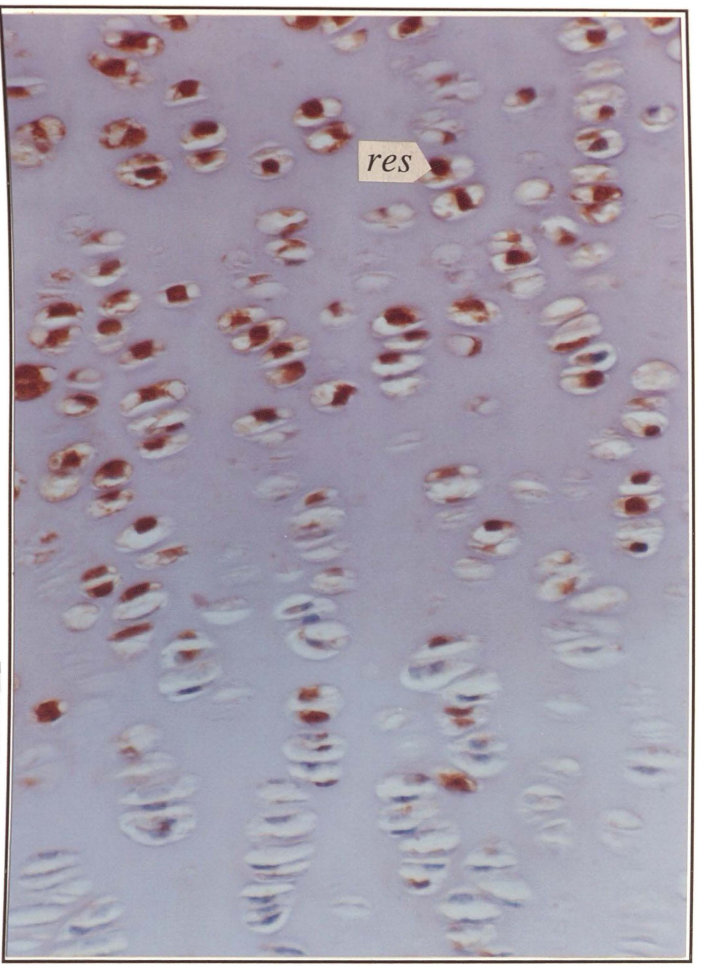
Bone sections from the proximal femur of either a 50 (a) or 124 (b) day gestation animal from the foetal ontogeny study analysed by immunohistochemistry.

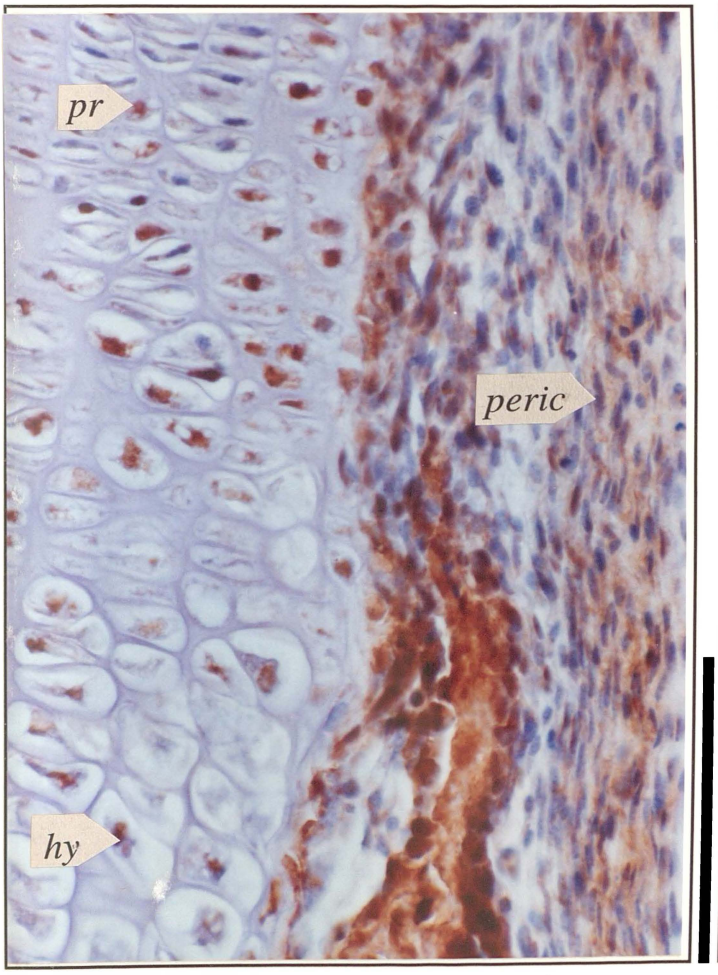
Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)

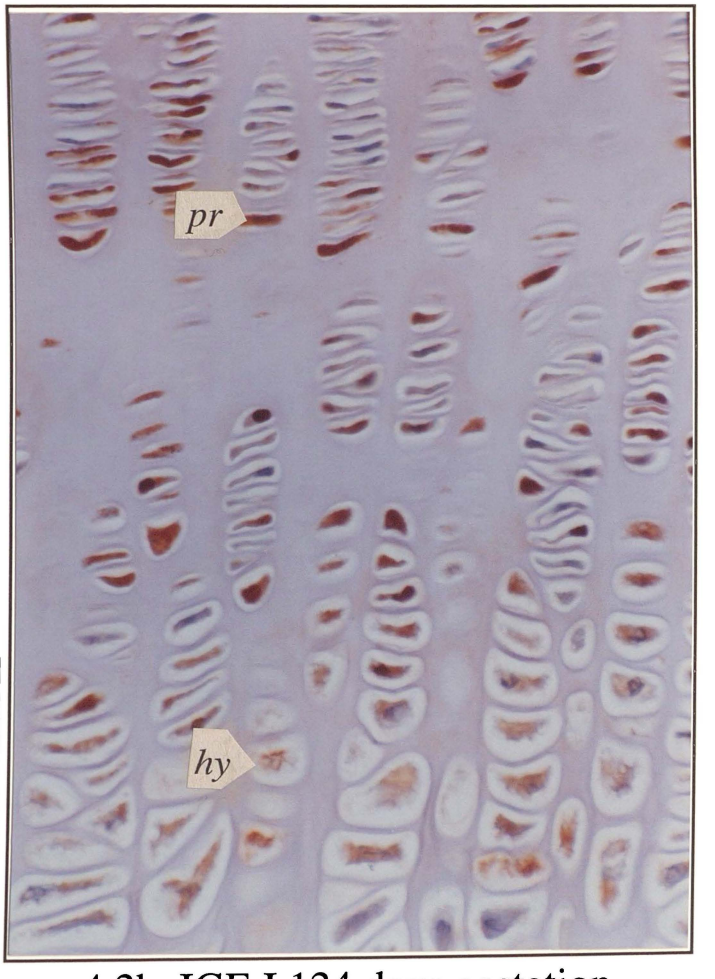


Positive immunostaining for IGF-I appears as a brown stain
peric, perichondrium; *res,* reserve chondrocyte*; pr*, proliferative chondrocyte; *hy*, hypertrophic chondrocyte
Vertical Bar = 100 μm









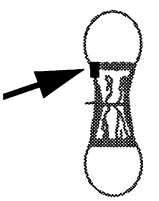
4.2a IGF-I 50 days gestation

4.2b IGF-I 124 days gestation

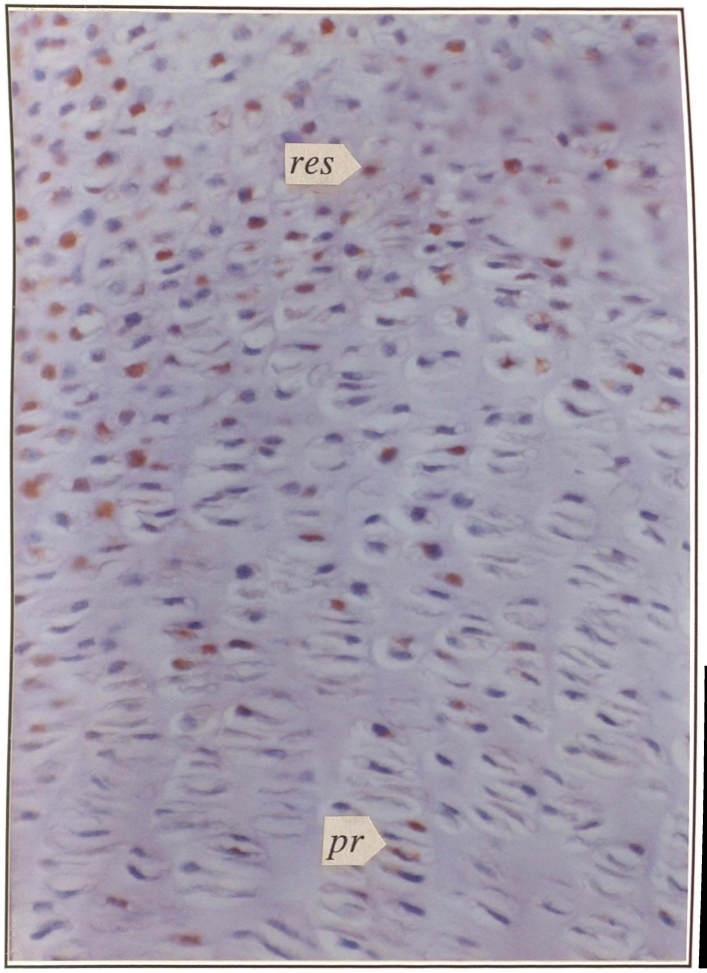
Figure 4.3 (a, b)
LOCALISATION OF IGF-II IN THE GROWTH PLATE

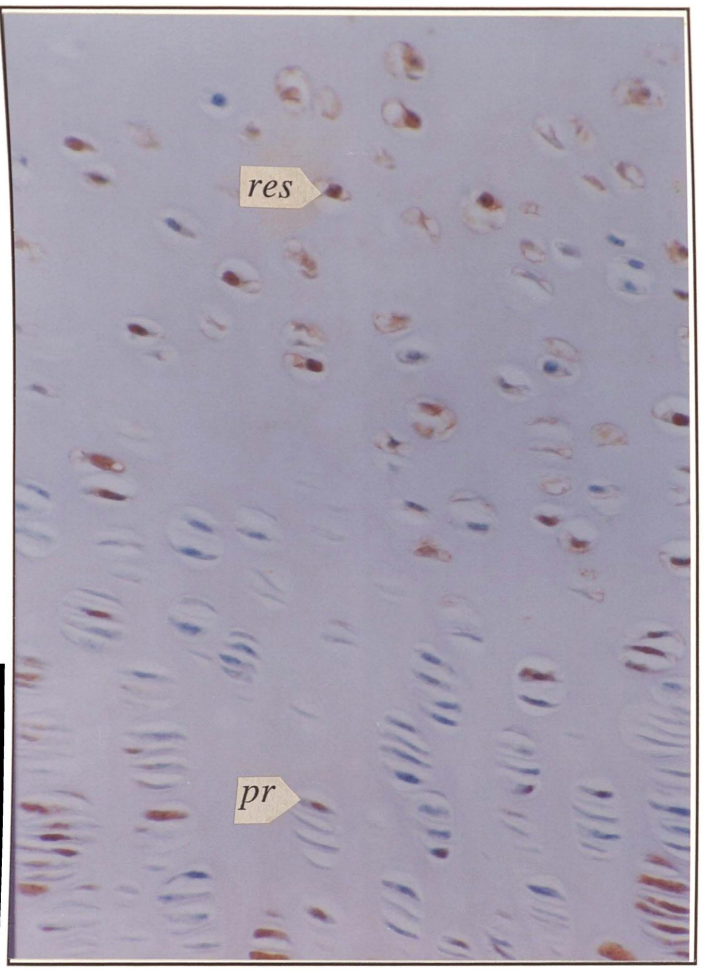
Bone sections from the proximal femur of either a 50 (a) or 124 (b) day gestation animal from the foetal ontogeny study analysed by immunohistochemistry.

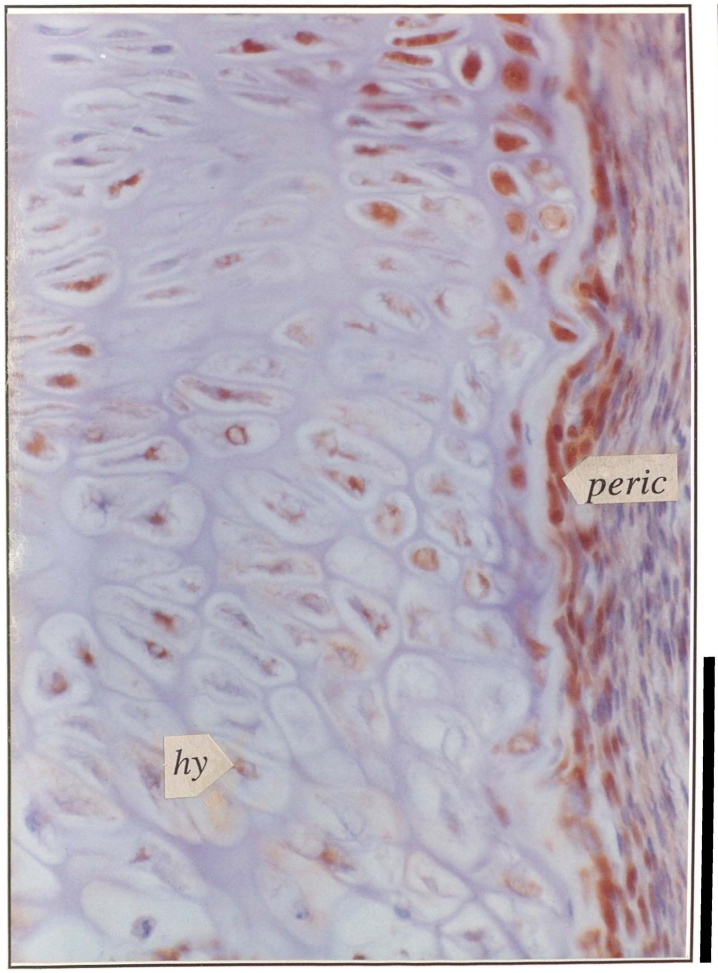
Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)

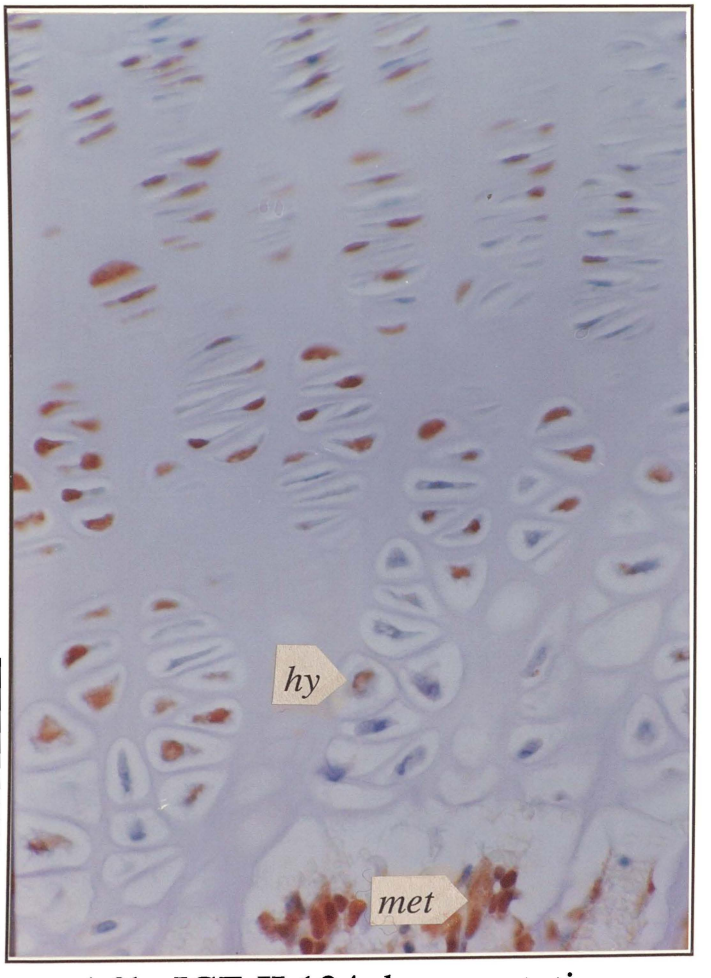


Positive immunostaining for IGF-II appears as a brown stain
peric, perichondrium; *res*, reserve chondrocyte; *pr*, proliferative chondrocyte; *hy*, hypertrophic chondrocyte; *met*, metaphysis
Vertical Bar = 100 μm









4.3a IGF-II 50 days gestation

4.3b IGF-II 124 days gestation

IGF-II, the number of stained cells in this zone varied over development, whereas for IGF-I a similar number of hypertrophic cells were stained at each age (Figures 4.2 and 4.3).

4.3.1.1.2 *Osteogenesis*

Both the matrix and cells within bone stained positively for IGF-I and IGF-II throughout foetal development (Figure 4.4). Staining in the matrix was observed in new and mature bone matrix regardless of where it was located. Osteogenic cells, osteoblasts and osteocytes all stained for IGF-I and IGF-II. However, further localisation at the subcellular level was unable to be determined due to the difficulty in clearly identifying staining over the cytoplasm or nucleus in these cells.

Bone cells had a similar pattern of staining for IGF-I and IGF-II. In general, osteogenic cells residing in the periosteum were more intensely stained than those in bone cavities (Figure 4.4). Staining to these cells at low magnification appeared as a band in the inner layer of the periosteum (Figure 4.4).

Almost every osteoblast was stained at every age for IGF-I and IGF-II (Figure 4.4). Overall, osteoblasts were the most intensely stained cells in these sections, and were positive for IGF-I and IGF-II regardless of where they were located. Although not as well stained, most osteocytes embedded in bone matrix were positive for IGF-I and IGF-II (Figure 4.4).

The pattern of cell staining observed for osteogenic cells, osteoblasts and osteocytes in the diaphysis was also found for these cells in the secondary centre of ossification at the older ages (data not shown).

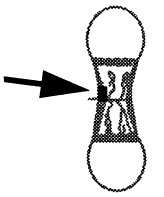
4.3.1.2 Western Analysis of IGF-I and IGF-II

Despite using a sensitive detection system, no bands could be detected by Western blot analysis for IGF-I (7.7 kDa), IGF-II (7.5 kDa) or IGFs present in their unprocessed pre-pro form (approximately 21 kDa) (Hill and De Sousa, 1990a) in growth plate proteins analysed with an IGF-I or IGF-II antibody, respectively (Figure 4.5). Some bands were observed in the 55 - 65 kDa range with the IGF-I antibody and 60 - 70 kDa range with the IGF-II antibody.

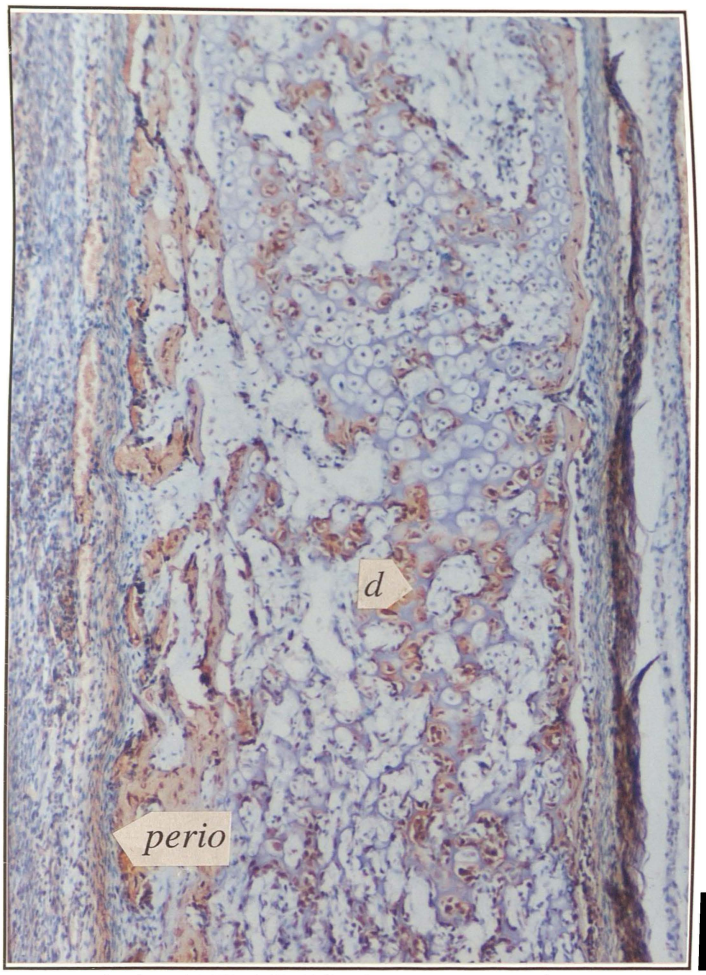
Figure 4.4 (a, b, c, d)
LOCALISATION OF IGF-I AND IGF-II IN THE DIAPHYSIS

Bone sections from the proximal femur of either 50 (a and c) or 124 (b and d) day gestation animals from the foetal ontogeny study analysed by immunohistochemistry. Comparison between IGF-I (a and c) and IGF-II (b and d) immunostaining.

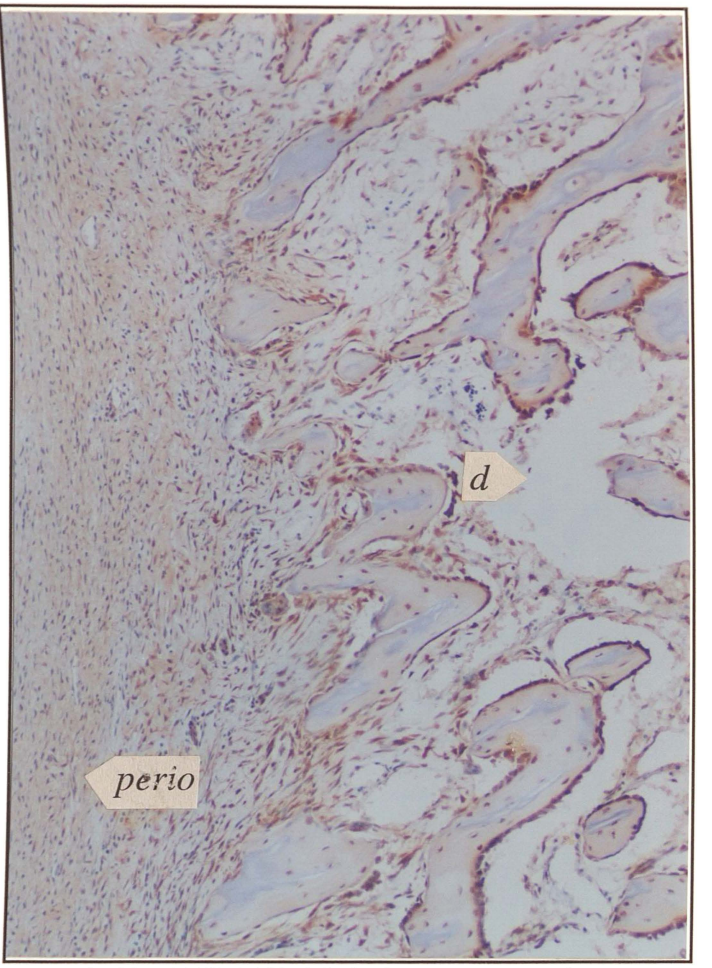
Photographs are orientated in the longitudinal plane:
(a view through the diaphysis)



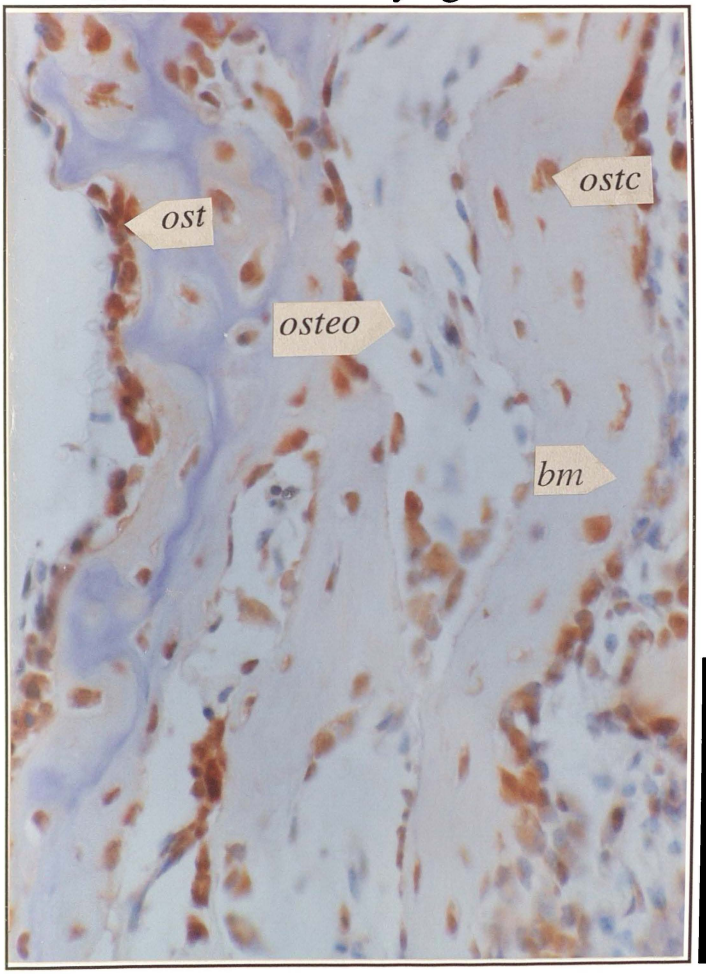
Positive immunostaining for IGF-I and IGF-II appears as a brown stain
perio, periosteum; *d*, diaphysis; *osteo*, osteogenic cell; *ost*, osteoblast, *ostc*, osteocyte; *bm*, bone matrix
Vertical Bar = 100 μm



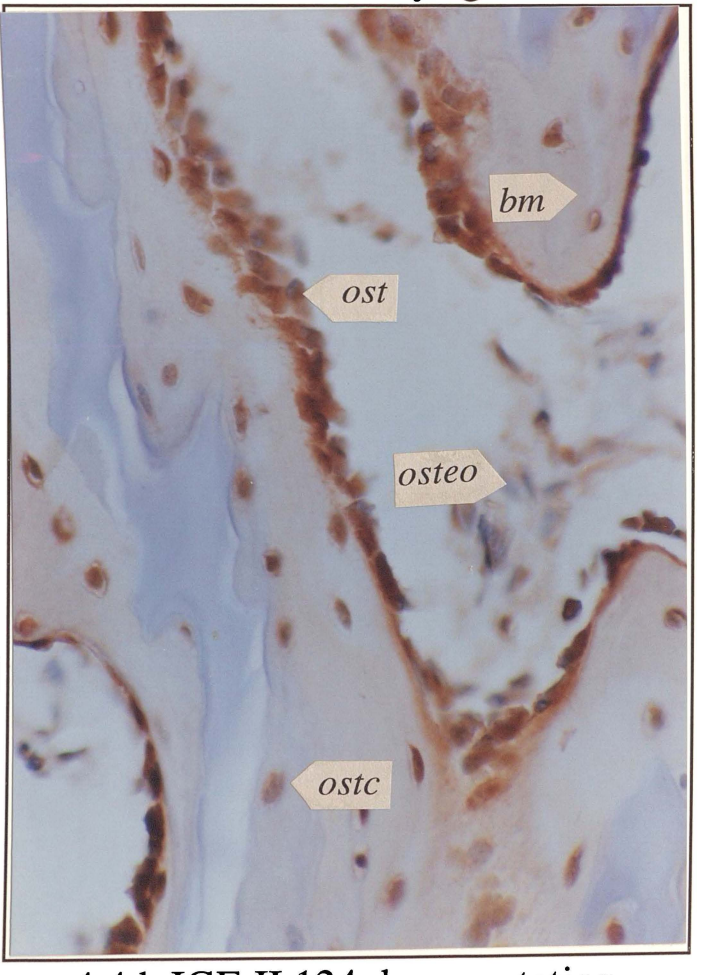
4.4a IGF-I 50 days gestation



4.4b IGF-II 124 days gestation



4.4c IGF-I 50 days gestation



4.4d IGF-II 124 days gestation

Although these bands could be due to IGF complexed to matrix proteins, it is more likely that they are the result of non-specific binding of antibody to IgGs present in the samples. Similar results have been obtained by others attempting to analyse IGFs in plasma and tissues (Bicsak *et al.*, 1990). These bands are unlikely to be due to IGF bound to IGF BPs since they do not fall within the range of sizes reported for IGF BPs in bone (20 - 43 kDa) even with IGF bound to them (Sunic *et al.*, 1995; Olney *et al.*, 1993; Froger-Gaillard *et al.* 1989).

IGF-I and IGF-II were also unable to be detected in other samples from the sheep foetus that were included in the analysis as a positive control (Figure 4.5). It is possible that the levels of IGF-I and IGF-II in all samples are beyond the level of detection using these particular antibodies. In a study investigating IGF-I and IGF-II content in various human foetal tissues, liver and skeletal muscle were found to contain barely detectable levels of IGF-I and only slightly higher levels of IGF-II (range 10 to 50 fM per 100 µg DNA (Hill and De Sousa, 1990a). Levels of IGF-I and IGF-II in human bone (contains bone and cartilage) were measured and found to be 84 µg/kg wet weight and 802 µg/kg wet weight, respectively (Frolik *et al.*, 1988). Based on these figures, the samples in the present study contained approximately 42 pg of IGF-I and 400 pg IGF-II which was below the level of detection. Neither 0.75 ng of IGF-I or IGF-II were able to be detected in the present study (Figure 4.5). Since IGF-I and IGF-II were found to be mostly located in cells by immunohistochemistry, it is likely that the extremely high protein content of the extracellular matrix in cartilage and bone diluted the contribution of cellular protein. Further investigation and optimisation of Western blotting is required. Improvements to enrich for cellular proteins and to dissociate IGFs from IGF BPs need to be carried out.

4.3.2 IGF Binding Sites in Foetal Bone Development

4.3.2.1 **Localisation of IGF-I Binding Sites**

Specific binding (from now on referred to as binding) of IGF-I to the growth plate was similar at 62, 103 and 140 days gestation (Table 4.3). While the hypertrophic zone displayed the highest binding, significant binding was also found in the proliferative zone (Figure 4.6).

		Specific IGF-I Binding			Specific IGF-II Binding		
		62 days gestation	**103 days gestation**	**140 days gestation**	**62 days gestation**	**103 days gestation**	**140 days gestation**
CELLS AND MATRIX	reserve zone	+	+	+	++	+	+
	proliferative zone	+++	++	++	++++	+++	+++
	hypertrophic zone	++++	++++	++++	+++	+++	+++
CELLS	reserve zone (chondrocytes)	–	+	+	–	–	+
	proliferative zone (chondrocytes)	+++	+	+	++++	++	++
	hypertrophic zone (chondrocytes)	+++	+	+	++	++	+
MATRIX	reserve zone (cartilage matrix)	+	–	–	++	+	–
	proliferative zone (cartilage matrix)	–	+	–	–	+	+
	hypertrophic zone (cartilage matrix)	+	++	+	+	+	++

Table 4.3

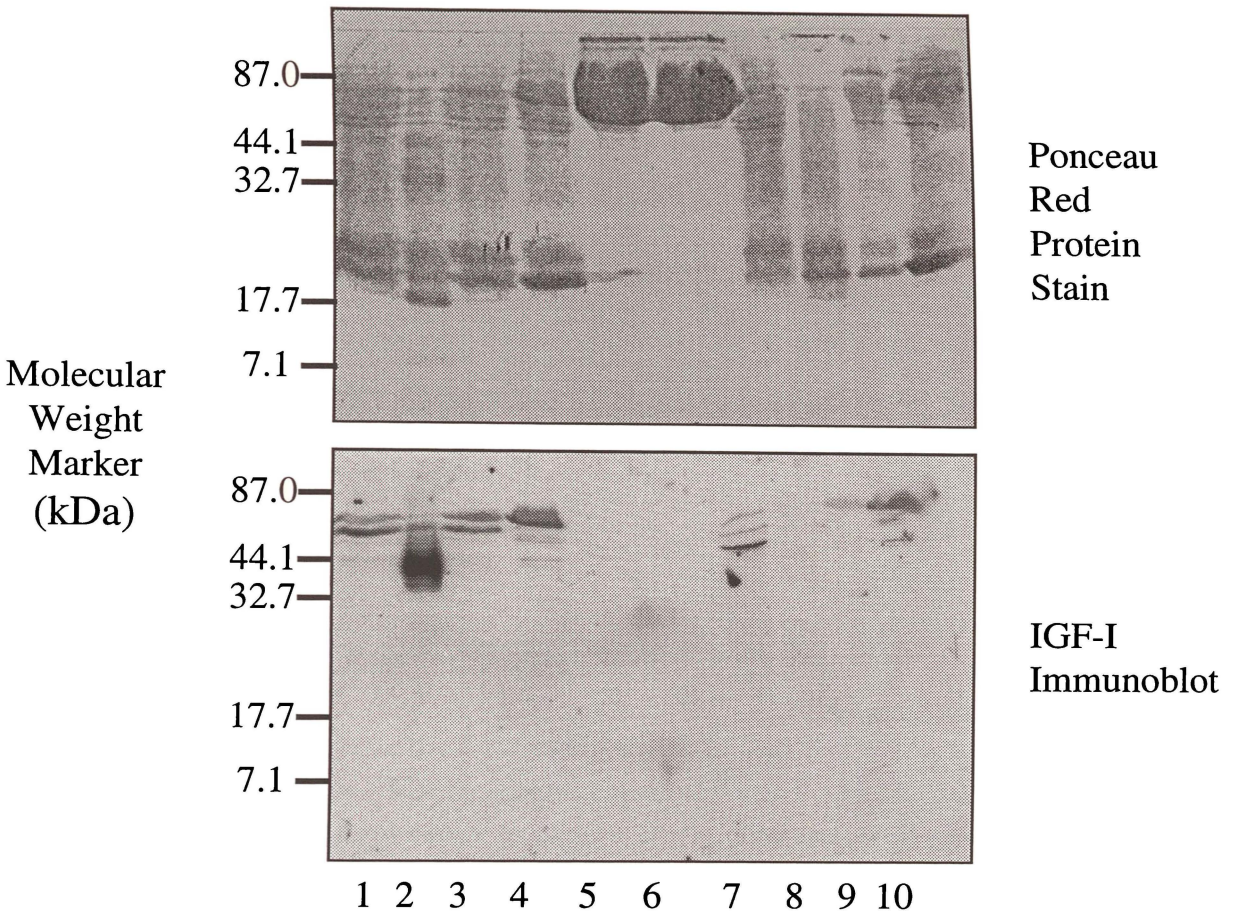
Summary of the localisation and relative abundance of IGF-I and IGF-II binding sites in different cells and regions of bone. The growth plates of three animals from each age were analysed.
Specific binding was assessed on a scale using arbitrary units from high (++++) to low (+). It reflects the difference between total binding (^{125}I-IGF) and non-specific binding (^{125}I-IGF in the presence of an excess of IGF)
(–) indicates an absence of specific binding

Figure 4.5 (a, b)

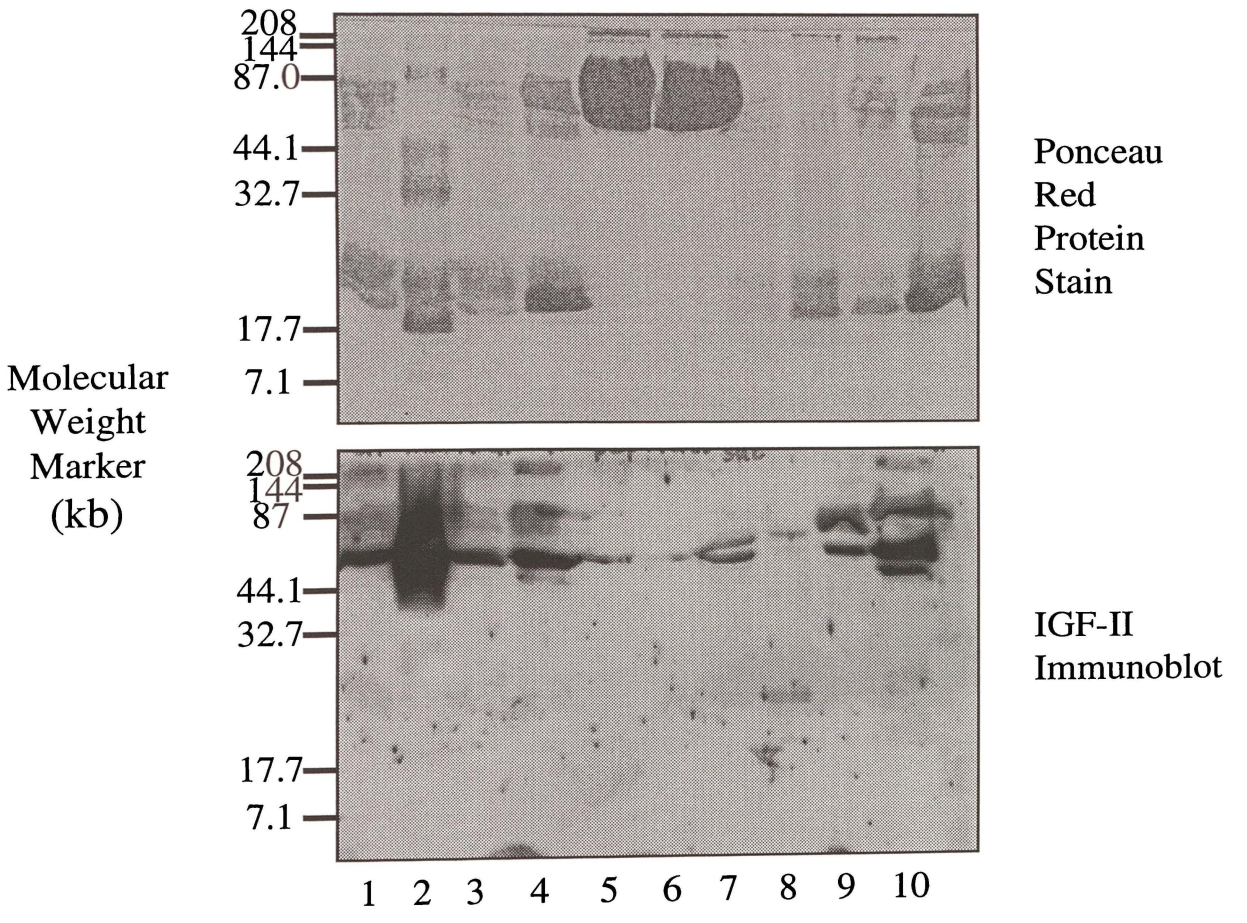
Protein extracted from the growth plate of the proximal humerus from 82 and 140 days gestation animals was analysed by Western blotting for IGF-I (a) and IGF-II (b). Protein was stained with Ponceau Red while immunoblot detection was by enhanced chemiluminescence.

Protein loaded in each lane was as follows:

1	82 days gestation, humerus (growth plate)
2	molecular weight marker
3	82 days gestation, humerus (growth plate)
4	140 days gestation, humerus (growth plate)
5	IGF-I (0.75 ng)
6	IGF-II (0.75 n g)
7	yolk sac, sheep 17 days gestation
8	foetal liver
9	foetal skeletal muscle
10	140 days gestation, humerus (growth plate)



4.5a IGF-I

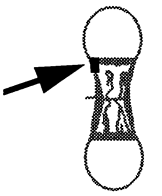


4.5b IGF-II

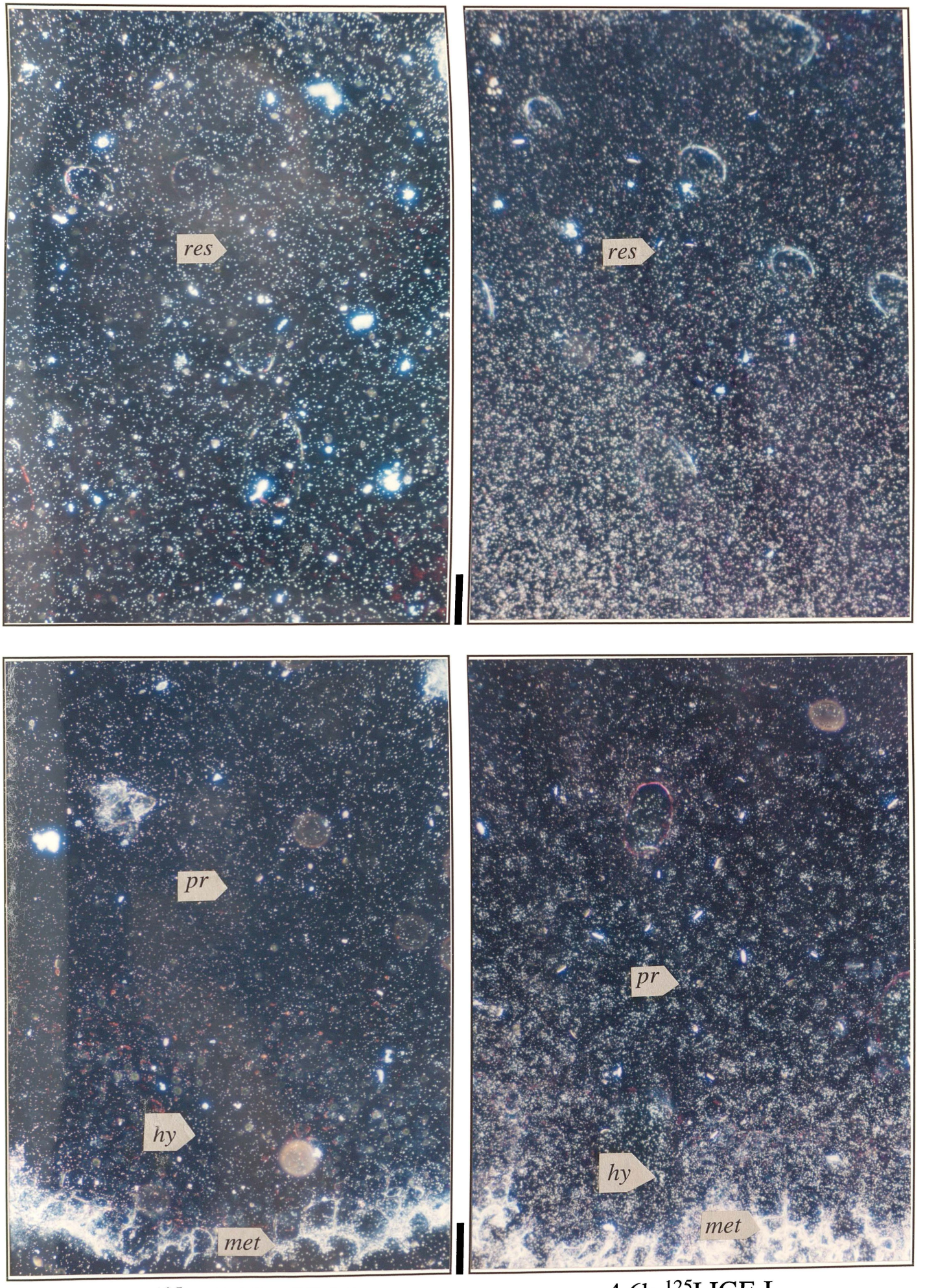
Figure 4.6 (a, b)
LOCALISATION OF IGF-I BINDING SITES

Bone sections from the proximal humerus of a 62 day gestation animal from the foetal ontogeny study. Photographs represent IGF-I binding sites in the reserve cartilage and hypertrophic zones of the growth plate with (a) and without (b) an excess of competing unlabelled IGF-I. Localisation of ^{125}I-IGF-I is visualised by dark field (white grains).
Exposure to emulsion was for 21 days.

Photographs are orientated in the longitudinal plane:
(a view through the growth plate)



res, reserve chondrocyte; *pr*, proliferative chondrocyte; *hy*, hypertrophic chondrocyte; *met*, metaphysis
Vertical Bar = 100 μm



4.6a ^{125}I IGF-I + IGF-I

4.6b ^{125}I IGF-I

IGF-I binding in the reserve cartilage zone was barely above background at any age (Figure 4.6).

IGF-I binding sites were largely localised to cells, however variable binding in the cartilage matrix in all zones was also observed (Table 4.3). Whilst binding to cells in the lower zones was prominent at 62 days gestation, there was little difference between zones at the older ages.

Binding of labelled IGF-I in the presence of an excess of IGF-II resulted in a decrease in binding of labelled IGF-I in all zones. However, binding was not reduced to the level of non-specific binding (data not shown). This suggests IGF-I is binding to a site(s) that IGF-II is also able to bind to. However, since an excess of IGF-II was unable to completely abolish IGF-I binding it is concluded that IGF-I binds to these site(s) with higher affinity. By comparison, insulin was unable to reduce the level of IGF-I binding in these sections, indicating that the observed binding of IGF-I was not to the insulin receptor (data not shown).

4.3.2.2 Localisation of IGF-II Binding Sites

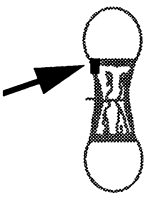
Specific binding of IGF-II to the growth plate (from now on referred to as binding) tended to be higher at 62 days gestation compared to 103 and 140 days gestation (Table 4.3). As was found for IGF-I binding, most IGF-II binding was localised to the lower zones (proliferative and hypertrophic) of the growth plate, with negligible binding in the reserve cartilage zone (Table 4.3 and Figure 4.7). Compared to IGF-I binding, there was a tendency for more binding in the cartilage matrix than over cells. Binding over cells was similar to that observed for IGF-I binding, where IGF-II binding was found mostly in the proliferative and hypertrophic zones (Figure 4.7).

Binding of labelled IGF-II in the presence of an excess of IGF-I resulted in a slight increase in binding (data not shown). A failure to decrease binding suggests that the IGF-II binding site(s) were specific for IGF-II and that IGF-I had very low affinity for them. However, the increase in binding indicates a more complex interaction between IGF-I and IGF-II was

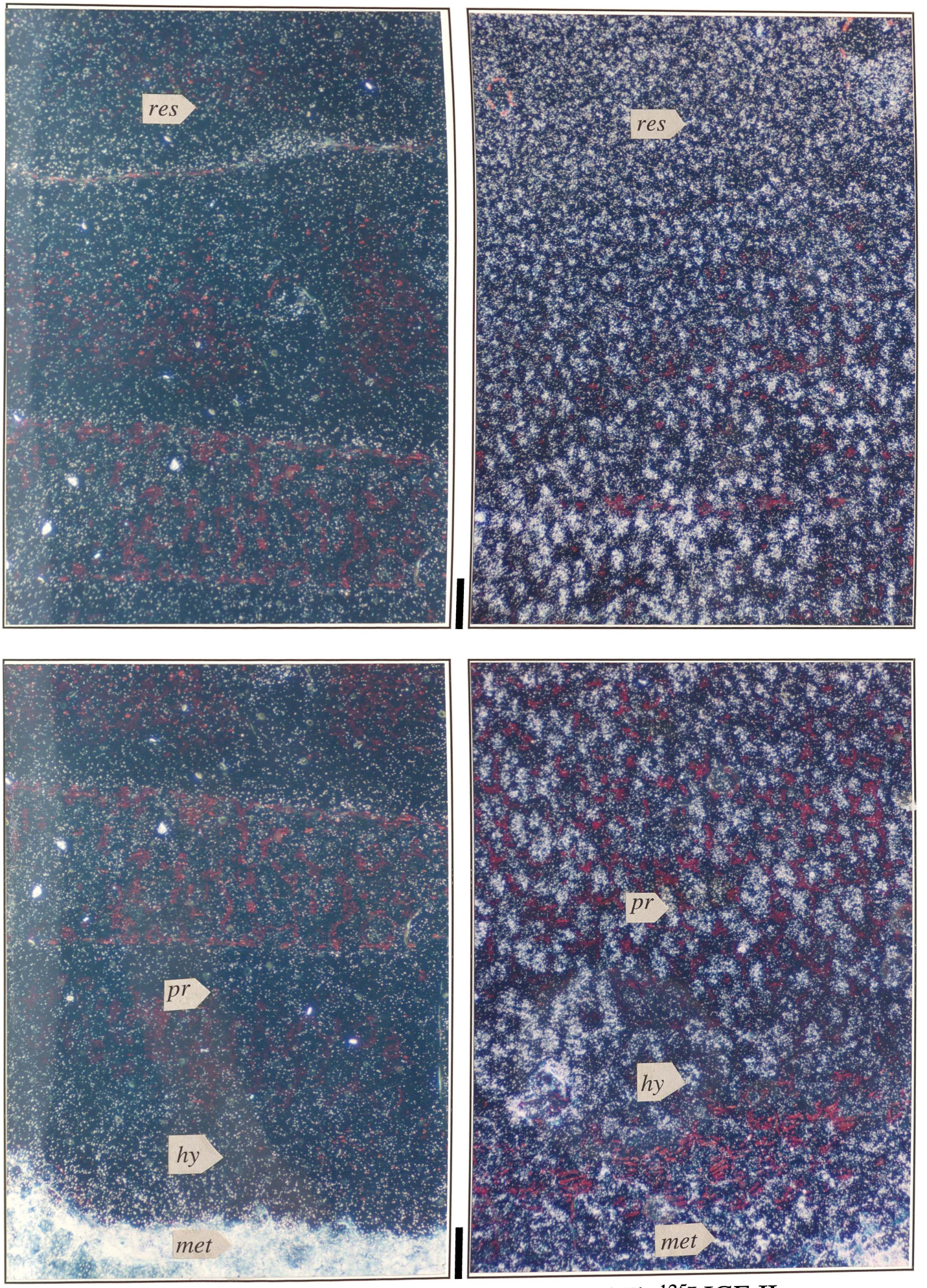
Figure 4.7 (a, b)
LOCALISATION OF IGF-II BINDING SITES

Bone sections from the proximal humerus of a 62 day gestation animal from the foetal ontogeny study. Photographs represent IGF-II binding sites in the reserve cartilage and hypertrophic zones of the growth plate with (a) and without (b) an excess of competing unlabelled IGF-II. Localisation of ^{125}I-IGF-II is visualised by dark field (white grains).
Exposure to emulsion was for 14 days.

Photographs are orientated in the longitudinal plane:
(a view through the growth plate)



res, reserve chondrocyte; *pr*, proliferative chondrocyte; *hy*, hypertrophic chondrocyte; *met*, metaphysis
Vertical Bar = 100 μm



4.7a ^{125}I IGF-II + IGF-II

4.7b ^{125}I IGF-II

occurring in these sections (see discussion). Insulin was unable to reduce the level of IGF-II binding (data not shown) suggesting that IGF-II binding was not to the insulin receptor.

4.3.2.3 Presence of Type I and Type II IGF Receptors in the Growth Plate

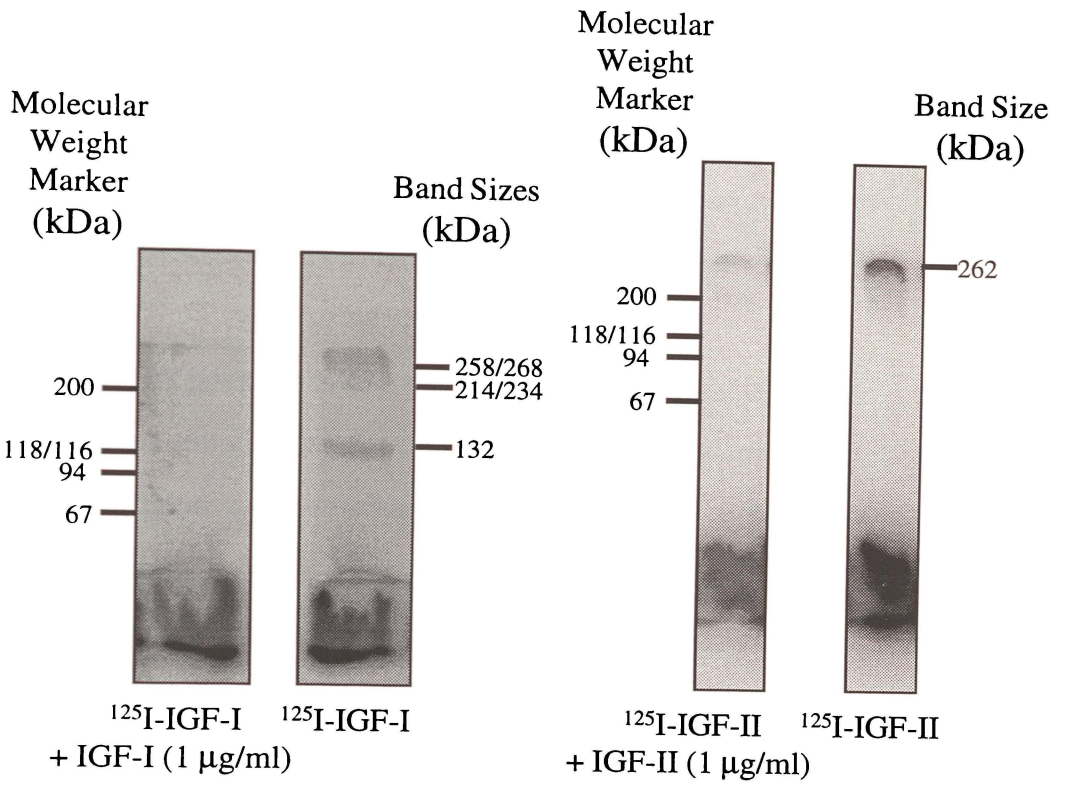
Under reducing conditions IGF-I was cross-linked predominantly to a 132 kDa species, however faint bands corresponding to cross-linked species of 231 and 259 kDa were also observed (Figure 4.8). The intensity of all three bands was reduced in the presence of an excess of unlabelled IGF-I indicating that they were specific for complexes containing IGF-I (Figure 4.8).

The 132 kDa species was indicative of IGF-I bound to the α subunit of the Type I IGF receptor, since not only has this size been previously reported for the α subunit (130 - 140 kDa), but it is within the α subunit of the Type I IGF receptor that IGF-I binding is thought to occur (Gustafson and Rutter, 1990; Trippel *et al.*, 1988; Ballard *et al.*, 1988; Ullrich *et al.*, 1986; Beguinot *et al.*, 1985).

The fainter bands were likely to be the result of IGF-I binding to the reduced (258 - 268 kDa) and non-reduced (214 - 234 kDa) forms of the Type II IGF receptor. Similar sized bands have previously been reported in IGF-I cross-linking studies (Elliott *et al.*, 1992; Ballard *et al.*, 1988; Trippel *et al.*, 1988).

IGF-II was found to cross-link to only one species at 262 kDa, under reducing conditions (Figure 4.8). In the presence of an excess of unlabelled IGF-II, binding to this species was abolished, indicating that this band corresponded to a complex containing IGF-II (Figure 4.8). The 262 kDa IGF-II cross-linked product was consistent with the size reported for the reduced form of the Type II IGF receptor (Ballard *et al.*, 1988; Trippel *et al.*, 1988).

Overall, it was concluded from these results that IGF-I binding was not only to the Type I IGF receptor, but also to the Type II IGF receptor. In contrast, IGF-II binding was specific for the Type II IGF receptor.



4.8a cross-linked IGF-I

4.8b cross-linked IGF-II

Figure 4.8 (a, b)

Crude microsomal membranes were prepared from a growth plate microdissected from a 140 day gestation humerus. 100 µg of protein was affinity cross-linked to either ^{125}IGF-I (a) or ^{125}IGF-II (b) in the presence of an excess (1 µg/ml) of unlabelled IGF-I or IGF-II, respectively. Affinity cross-linked products were electrophoretically separated on a 7.5% SDS polyacrylamide gel.
Exposure to X-ray film was for 3 days.

4.3.2.4 Distribution of IGF Binding Proteins -2, -3, -4 and -5

IGF BPs were detectable in all bone sections from the foetal ontogeny study with all IGF BPs displaying a similar distribution. All binding proteins were found to be similarly distributed at the cellular level (Tables 4.4 to 4.7). Overall, the pattern observed at 50 days gestation was similar across all the foetal ages studied (Tables 4.4 to 4.7). In general, IGF BPs were less prominent in cartilage compared to bone. In cartilage, IGF BPs were confined to particular cells within specific zones, throughout development. In bone, staining was more widespread and was found both within cells and extracellularly in the bone matrix.

Although it was not possible to test the specificity of each antibody for their respective IGF BPs, specifications reported by the company and other researchers indicate that IGF BP-2 antibody has negligible cross-reactivity with other IGF BPs (Clemmons *et al.*, 1991; Upstate Biotechnology Incorporated, 1995). Similarly, IGF BP-3 and IGF BP-5 antibodies have minimal cross-reactivity with other IGF BPs (Upstate Biotechnology Incorporated, 1995). However, interpreting results with the IGF BP-4 antibody must be done with caution, since up to 50% cross reactivity with IGF BP-2 has been reported using this antibody (Upstate Biotechnology Incorporated, 1995; Camacho-Hubner *et al.*, 1992).

4.3.2.4.1 *Chondrogenesis*

Immunostaining in cartilage was exclusively localised to cells and not to the cartilage matrix (Figure 4.9). In some cells staining was specific to the cytoplasm or nucleus whereas other cells stained both over the nucleus and the cytoplasm (data not shown). The lack of staining in cartilage matrix was found for all ages and IGF BPs, regardless of whether the cartilage was located on the articular surface, growth plate or within bone (cartilage trabeculae) (Figure 4.9).

Within cells in the growth plate, a similar distribution of staining for all IGF BPs and foetal ages was found (Figure 4.9). IGF BPs were largely localised to the hypertrophic and reserve cartilage zones, with most chondrocytes staining positively in these areas. Unstained cells

IGF BP-2	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	++	++	+	+	n.d.	n.d.
reserve zone (chondrocytes)	+	+	++	+	+++	+++
proliferative zone (chondrocytes)	+	–	+	–	+	+
hypertrophic zone (chondrocytes)	++	+++	+	+	++	+
Cartilage Matrix	–	–	–	–	–	–
periosteum (osteogenic cells)	++	++	++	+	++	++
bone cavity (osteogenic cells)	+	+	+	–	+	+
osteoblasts	++++	++++	++++	++++	++++	++++
osteocytes	+++	+++	+++	+++	+++	+++
Bone Matrix	++	++	++	++	++	++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	++++	++++
muscle fibres	++++	++++	+++	n.d.	n.d.	n.d.

Table 4.4

Summary of the localisation and relative abundance of immunoreactive IGF BP-2 in different cells and regions of bone. Bone sections from one animal per age from the foetal ontogeny study were analysed.
Specific immunostaining was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where immunostaining was not determined; (–) indicates an absence of specific immunostaining.

IGF BP-3	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	+++	++	++	++	n.d.	n.d.
reserve zone (chondrocytes)	+	++	++	++	+++	+++
proliferative zone (chondrocytes)	–	–	–	–	+	+
hypertrophic zone (chondrocytes)	+++	+++	+	+	++	+
Cartilage Matrix	–	–	–	–	–	–
periosteum (osteogenic cells)	+++	+++	++	++	++	++
bone cavity (osteogenic cells)	++	+	+	+	+	++
osteoblasts	++++	++++	++++	++++	++++	++++
osteocytes	+++	++	++	++	++	+++
Bone Matrix	++	++	++	++	++	++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	++++	++++
muscle fibres	++++	++	++	n.d.	n.d.	n.d.

Table 4.5

Summary of the localisation and relative abundance of immunoreactive IGF BP-3 in different cells and regions of bone. Bone sections from one animal per age from the foetal ontogeny study were analysed.
Specific immunostaining was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where immunostaining was not determined; (–) indicates an absence of specific immunostaining.

IGF BP-4	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	–	–	+	+	n.d.	n.d.
reserve zone (chondrocytes)	+	++	+++	+++	+++	+++
proliferative zone (chondrocytes)	–	+	+	+	+	–
hypertrophic zone (chondrocytes)	++	+++	+++	+++	++	++
Cartilage Matrix	–	–	–	–	–	–
periosteum (osteogenic cells)	++	++	+++	++	++	++
bone cavity (osteogenic cells)	+	–	+	+	+	+
osteoblasts	++++	++++	++++	++++	++++	++++
osteocytes	++	+++	+++	++	++	++
Bone Matrix	+	++	++	++	++	++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	++++	++++
muscle fibres	+++	+++	n.d.	n.d.	n.d.	n.d.

Table 4.6

Summary of the localisation and relative abundance of immunoreactive IGF BP-4 in different cells and regions of bone. Bone sections from one animal per age from the foetal ontogeny study were analysed.
Specific immunostaining was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where immunostaining was not determined; (–) indicates an absence of specific immunostaining.

IGF BP-5	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	++	++	+	+	n.d.	n.d.
reserve zone (chondrocytes)	+	+++	+++	+++	+++	+++
proliferative zone (chondrocytes)	–	+	+	+	++	+
hypertrophic zone (chondrocytes)	+++	+++	++	+++	++	++
Cartilage Matrix	–	–	–	–	–	–
periosteum (osteogenic cells)	+++	++	++	++	+	+++
bone cavity (osteogenic cells)	++	++	++	++	+	++
osteoblasts	++++	++++	++++	++++	++++	++++
osteocytes	+++	++++	+++	+++	++	+++
Bone Matrix	+	++	++	++	++	++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	++++	++++
muscle fibres	+++	+++	++++	n.d.	n.d.	n.d.

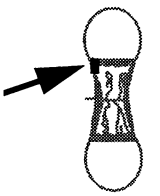
Table 4.7

Summary of localisation and relative abundance of immunoreactive IGF BP-5 in different cells and regions of bone. Bone sections from one animal per age from the foetal ontogeny study were analysed.
Specific immunostaining was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where immunostaining was not determined; (–) indicates an absence of specific immunostaining.

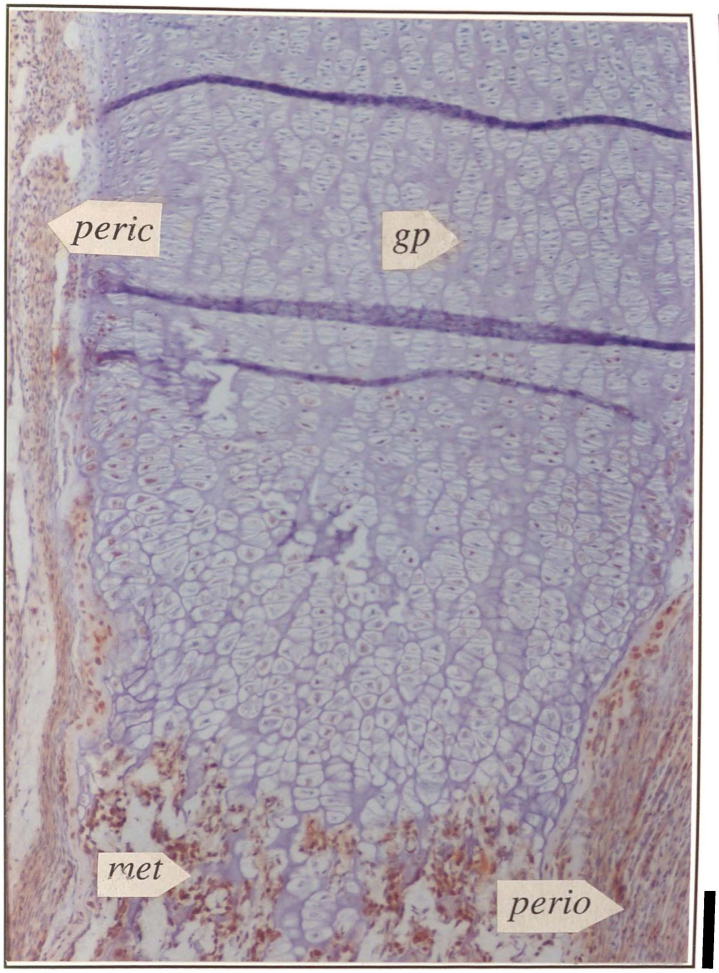
Figure 4.9 (a, b, c, d, e, f, g, h)
LOCALISATION OF IGF BP-2, -3, -4 AND -5 IN THE GROWTH PLATE

Bone sections from the proximal femur of either 50 (a, c, e and g) or 140 (b, d, f and h) day gestation animals from the foetal ontogeny study analysed by immunohistochemistry. Sections were analysed for either IGF BP-2 (a and b), IGF BP-3 (c and d), IGF BP-4 (e and f) or IGF BP-5 (g and h).

Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)



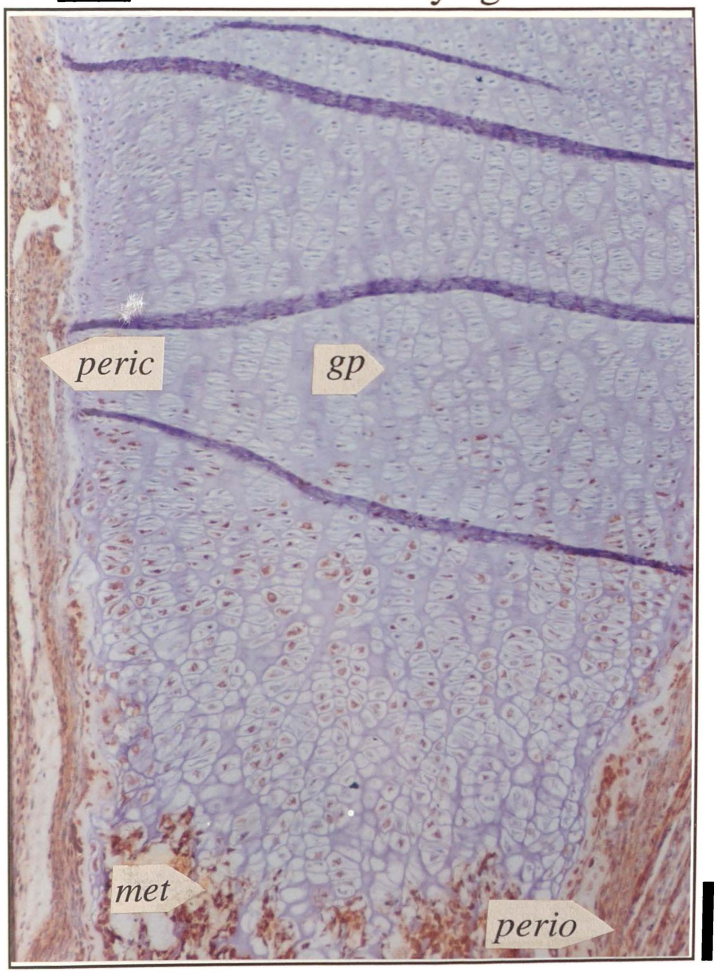
Positive immunostaining for each IGF BP appears as a brown stain
perio, periosteum; *peric*, perichondrium; *gp*, growth plate; *met*, metaphysis
Vertical Bar = 100 μm



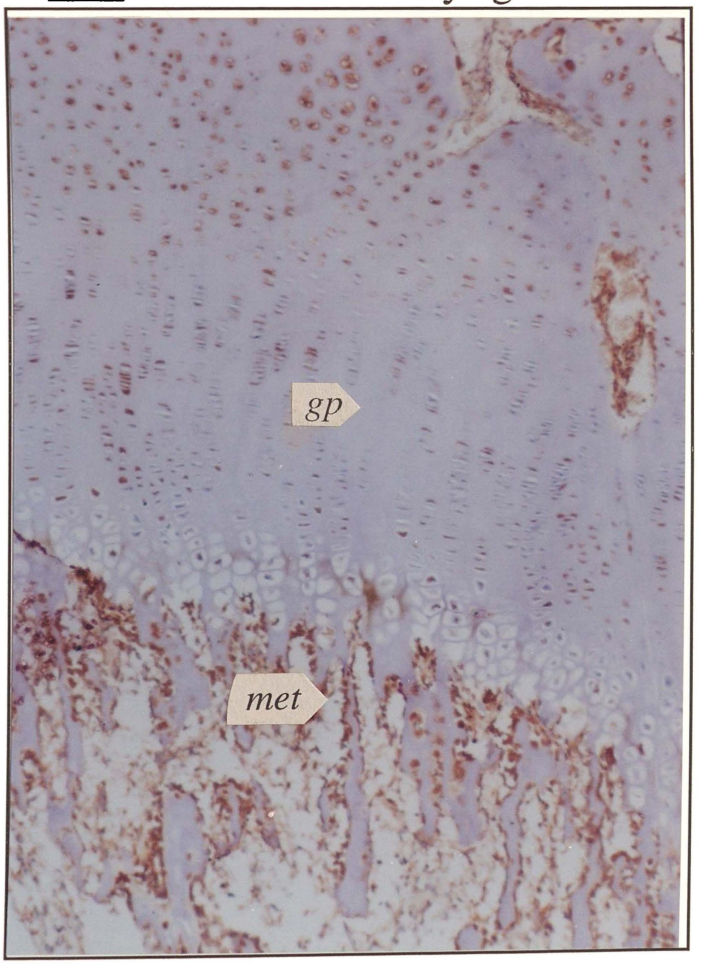
4.9a IGF BP-2 50 days gestation



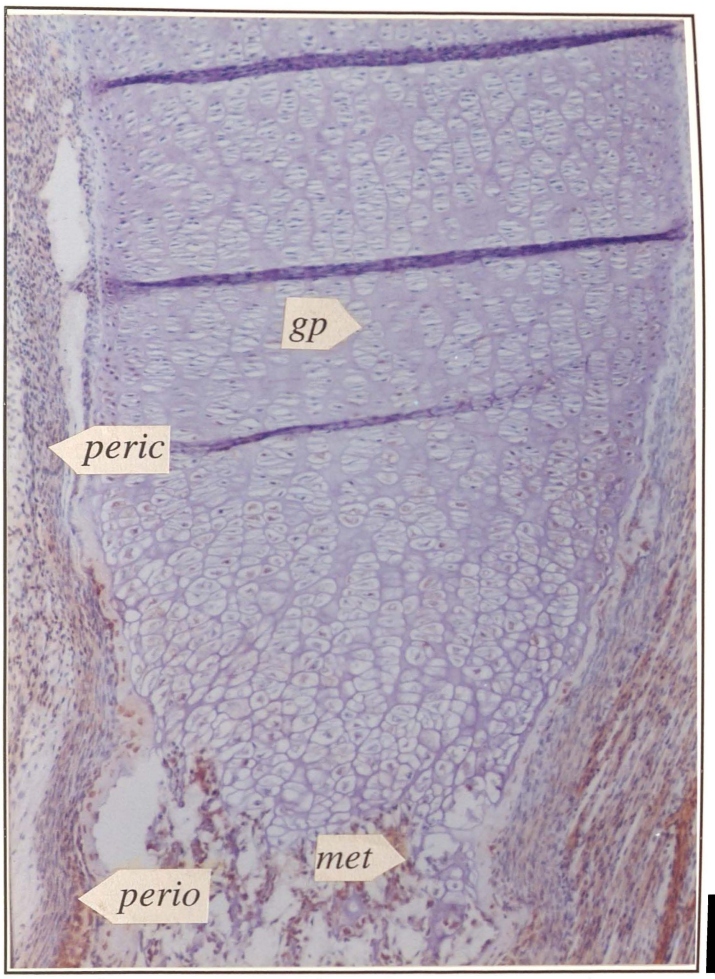
4.9b IGF BP-2 140 days gestation



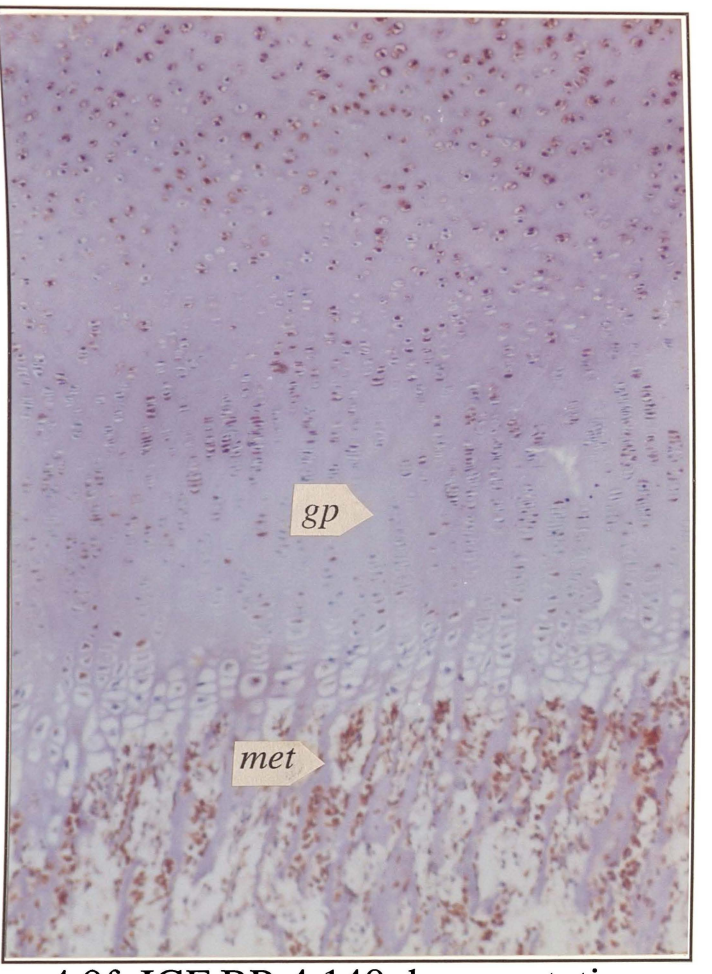
4.9c IGF BP-3 50 days gestation



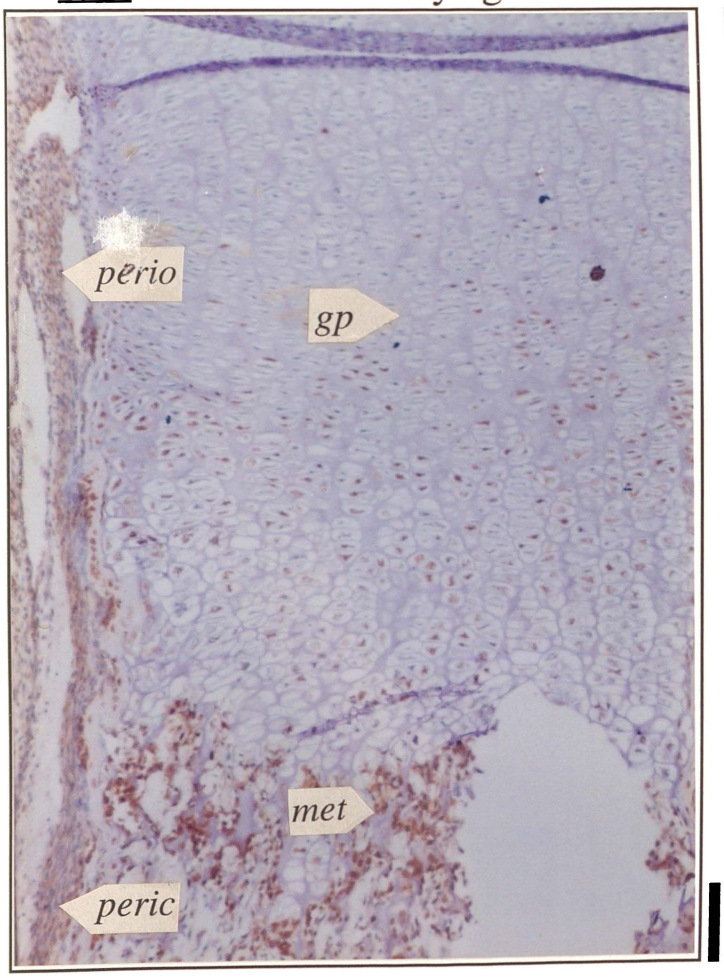
4.9d IGF BP-3 140 days gestation



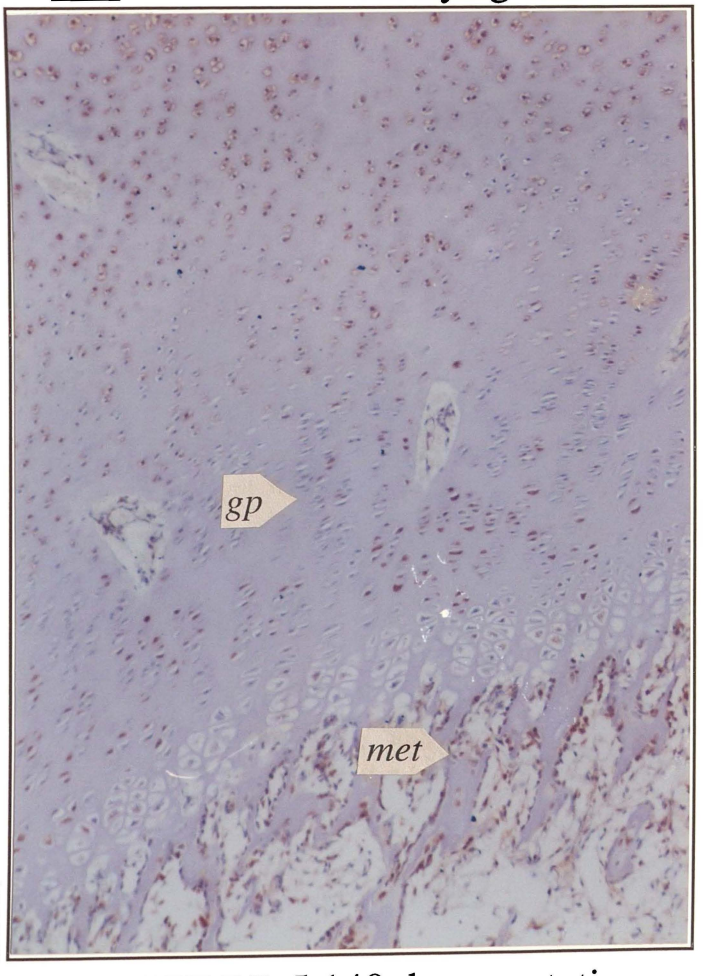
4.9e IGF BP-4 50 days gestation



4.9f IGF BP-4 140 days gestation



4.9g IGF BP-5 50 days gestation



4.9h IGF BP-5 140 days gestation

were often found alongside stained cells and could not be distinguished by their location or appearance from stained cells.

There was some variation in the staining pattern observed within each zone. In both the reserve cartilage and hypertrophic zones staining tended to be localised to the more peripherally located chondrocytes than to chondrocytes located in the centre of the growth plate. This was more noticeable at the younger ages (Figure 4.9).

Depending on the age and particular IGF BP, the last layer of hypertrophic cells adjacent to the metaphysis were either stained, as was found for IGF BP-4 at the earlier foetal ages, or not stained as was found for IGF BP-2. Similarly, for some ages and IGF BPs, more cells in the upper layer of the hypertrophic zone (maturation zone) were stained than the lower layers. This was found for IGF BP-2 at all ages and at the older foetal ages using the IGF BP-4 antibody. Since the IGF BP-4 antibody is predicted to cross react with IGF BP-2, the difference in immunostaining between IGF BP-2 and IGF BP-4 antibodies is likely to represent staining for IGF BP-4. In the reserve cartilage zone, staining was confined to only a few cells at the younger foetal ages. With increasing age there was an increase in the number of positively stained cells in this zone (Figure 4.9). Chondrocytes in the proliferative zone displayed very little staining for any IGF BP, at any age. Staining was only occasionally observed in a single chondrocyte within a column of chondrocytes (Figure 4.9).

At the earlier ages, where a defined perichondrium was able to be identified, weak staining was observed in the inner layer of the perichondrium (Figure 4.9). Staining to chondrogenic cells within this zone either decreased with foetal age (IGF BP-2, -3 and -5) or was not detected until later in the foetal period (IGF BP-4).

4.3.2.4.2 *Osteogenesis*

In contrast to cartilage, staining in bone was observed not only over cells but also in the extracellular matrix (data not shown). Bone matrix staining was found for all IGF BPs and foetal ages. Both newly formed bone matrix near the growth plate and mature bone in the diaphysis displayed a similar intensity of staining.

Osteogenic cells, osteoblasts and osteocytes from the bone cell lineage displayed positive staining for all IGF BPs (Figure 4.9). Where it could be determined, staining was observed over both the cytoplasm and nucleus in bone cells. However it was difficult to discern the subcellular localisation of staining in most cells. While some cells had prominent nuclei with a shrunken cytoplasm, other cells had very small nuclei.

Between cell types the relative staining intensity differed. While the intensity of IGF BP staining in osteoblasts was high at all foetal ages, a relatively lower staining intensity was observed in other bone cells (Tables 4.4 to 4.7). Almost every osteoblast was stained, regardless of whether it was adjacent to the periosteum, in a bone cavity, or in mature or newly formed bone.

Compared to osteoblasts, a lower intensity of staining was observed in osteogenic cells residing in the periosteum. This staining appeared as a distinctive band of cells adjacent to bone in the inner layer of the periosteum (Tables 4.4 to 4.7). Osteogenic cells residing within bone cavities were generally less well stained than those located in the periosteum. At some stages of development, staining in these cells was absent (IGF BP-2 and -4) whereas at other stages the intensity of staining was similar to that observed for osteogenic cells residing in the periosteum (IGF BP-3 and -5). Osteocytes at most locations within bone also stained positively for each IGF BP, although with less intensity than was observed for osteoblasts (Figure 4.9).

Another area of bone that displayed widespread staining for IGF BPs was the secondary centre of ossification (at the older ages) (data not shown). As was found in the diaphysis, staining in this area was most prominent in osteoblasts and the bone matrix, with lower staining in osteogenic cells.

4.3.3 Local Production of IGF BP-2, -4 and -5

IGF BP-2, -4 and -5 mRNA could not be detected in bone by Northern or *in situ* hybridisation, at any foetal age using either ovine BP-2 and BP-5 probes or rat BP-2, BP-4 and BP-5 probes (Figures 4.10 and 4.11). Additionally, BP-2 mRNA was absent in postnatal mouse bone, when RNA was analysed by Northern hybridisation (Figure 4.10).

The integrity of the BP-2 probe was confirmed by its ability to bind to IGF BP-2 mRNA in adult sheep liver and foetal sheep skeletal muscle on a Northern blot (1.9 kb), and 1 day old rat liver by *in situ* hybridisation (Figures 4.10 and 4.11). The pattern of hybridisation and the size of the transcript in these positive tissues agrees with that which has previously been reported (Shimasaki and Ling, 1991b; Böni-Schnetzler *et al.*, 1990; Orlowski *et al.*, 1990). Similarly, the integrity of the BP-4 and BP-5 probes was confirmed by their ability to bind specifically to adult rat liver and adult rat kidney, respectively (Figures 4.10 and 4.11). The pattern of hybridisation observed in these positive tissues was similar to that which has previously been reported (Shimasaki and Ling, 1991b; Shimasaki *et al.*, 1991a).

4.4 DISCUSSION

4.4.1 Localisation and Ontogeny of IGF Components in Foetal Growth Plate Cartilage

Overall, IGF-I, IGF-II and IGF BPs -2, -3, -4 and -5 were found to be similarly distributed at all stages of foetal development in chondrocytes from the hypertrophic and reserve cartilage zones of the growth plate. In the hypertrophic zone this pattern of localisation coincided with IGF-I and IGF-II binding, while in the proliferative zone IGF binding was found in the presence of low levels of IGF and IGF BPs. IGF and IGF BPs were abundant but IGF binding was very low in the reserve cartilage zone. Cellular binding of IGF-I and IGF-II represented binding to the Type I and Type II IGF receptors, respectively, whereas binding in the cartilage matrix, although not characterised, was likely to be to extracellular matrix components and/or

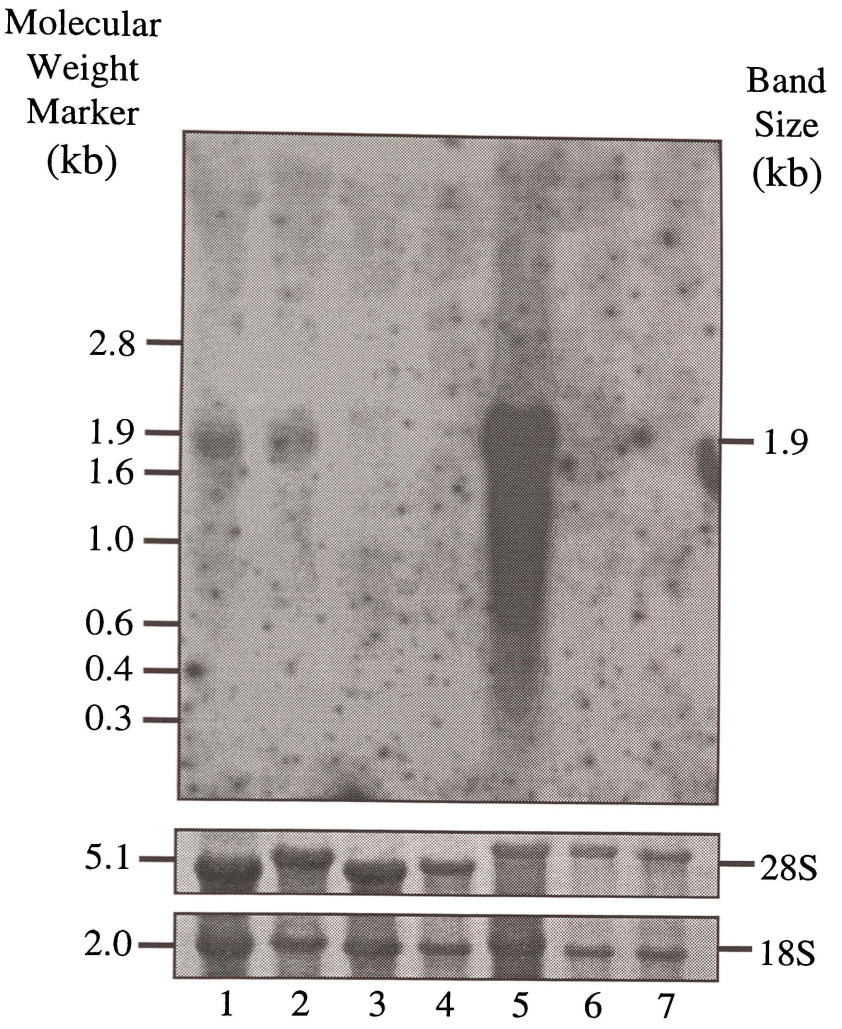


Figure 4.10

Northern blot of IGF BP-2. No IGF BP-2 mRNA was found in bone samples microdissected from either the diaphysis or growth plate of a 124 days gestation animal or bone from postnatal mice. IGF BP-2 mRNA was present in foetal skeletal muscle and adult sheep liver.
Total RNA samples (20 µg) were hybridised with an ovine IGF BP-2 probe. Lower panel shows hybridisation with a ribosomal RNA probe.

RNA loaded in each lane is as follows:

1	foetal skeletal muscle (20 µg)
2	foetal skeletal muscle (10 µg)
3	postnatal mouse bone (hind limb)
4	postnatal mouse bone (fore limb)
5	adult sheep liver
6	124 days gestation, humerus (diaphysis)
7	124 days gestation, humerus (growth plate)

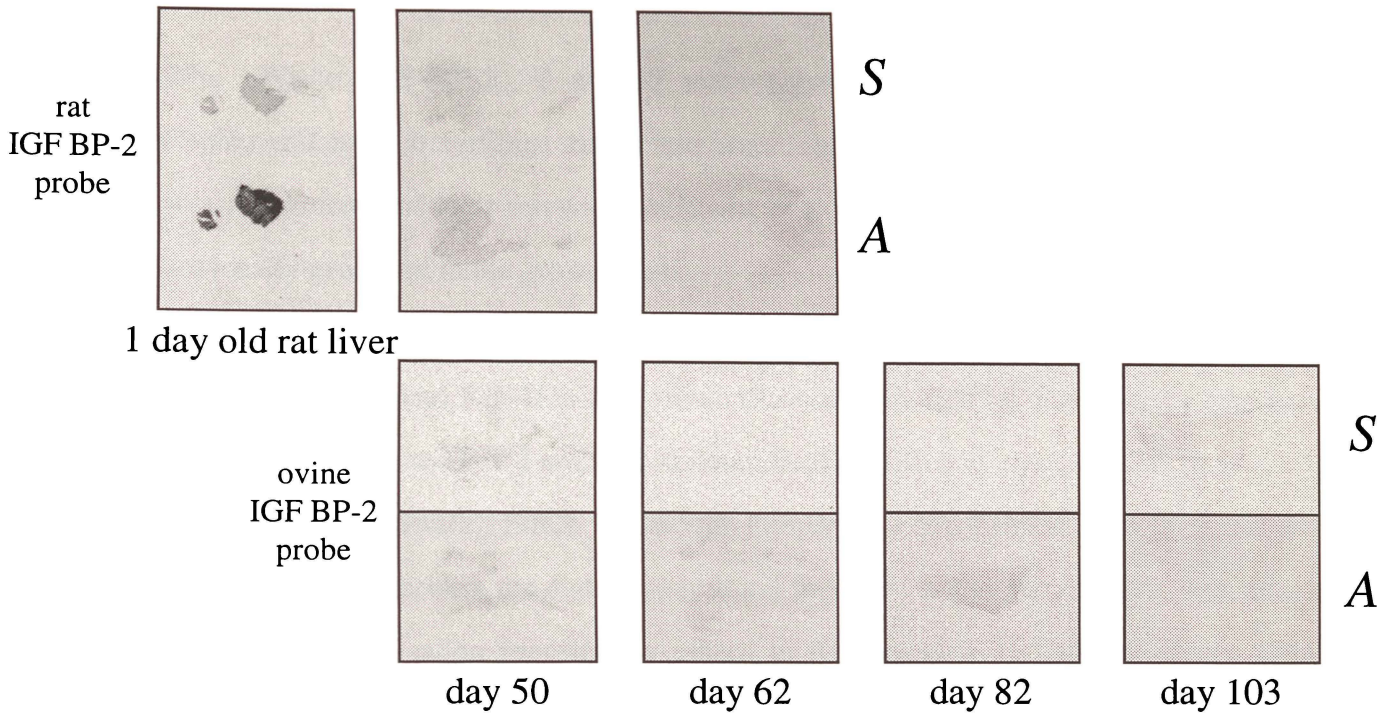
Blot was exposed for 7 days

Figure 4.11

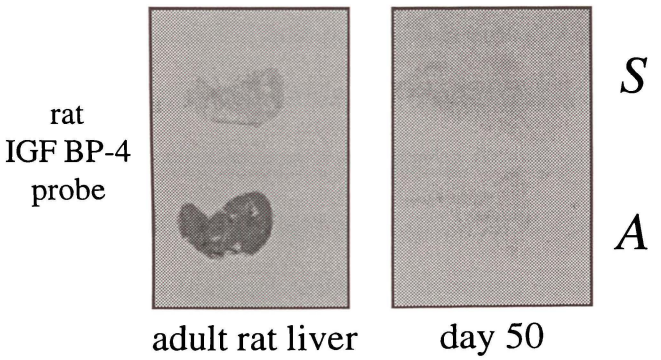
Autoradiographic images of bone sections from the foetal ontogeny study probed with either an antisense or sense rIGF BP-2 (a), oIGF BP-2 (a), rIGF BP-4 (b), rIGF BP-5 (c) or oIGF BP-5 (c) probe.
Rat and sheep probes are both presented to demonstrate that the inability to detect IGF BP mRNA in sheep bone was not due to species differences in the sequence of probe.
X-ray film exposure ranged from 3 to 7 days (depending on probe).

A represents antisense hybridisation
S represents sense hybridisation in adjacent sections

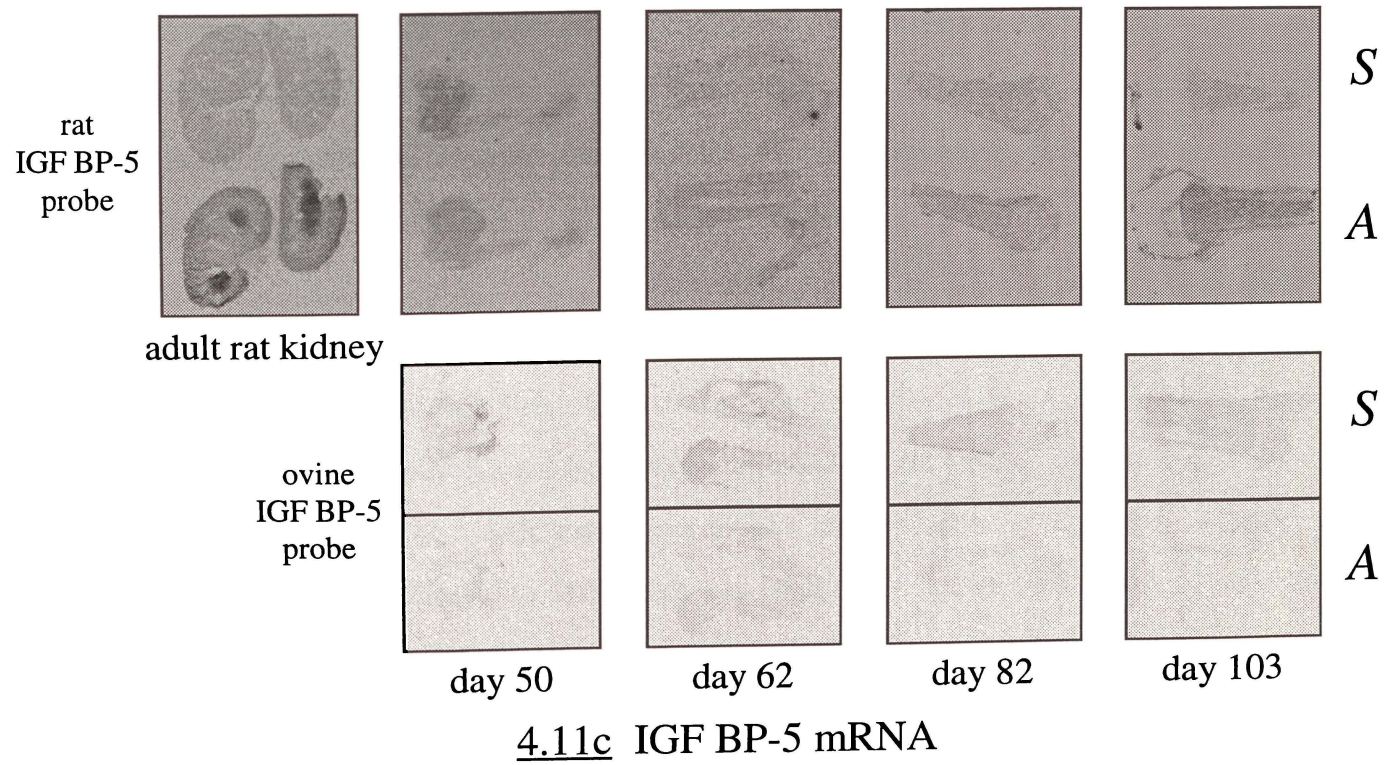
1 day old rat liver (IGF BP-2), adult rat liver (IGF BP-4) and adult rat kidney (IGF BP-5), previously shown to contain mRNA for the respective IGF BPs, are presented for comparison.



4.11a IGF BP-2 mRNA



4.11b IGF BP-4 mRNA



4.11c IGF BP-5 mRNA

IGF BPs. While IGF BP-2, -4 and -5 were present in most chondrocytes from the hypertrophic and reserve cartilage zones and some chondrocytes in the proliferative zone, there was no evidence of their respective mRNAs in any cell, suggesting that these IGF BPs were not locally produced in the growth plate.

The detection of IGF-I and IGF-II in the growth plate is supported by other studies that have extracted and characterised IGF-I and IGF-II from cartilage (Mohan *et al.*, 1988a; Kato *et al.*, 1981; Klagsbrun and Smith, 1980). Although the precise localisation of IGF-II has not previously been characterised, the distribution found for IGF-I in the growth plate was similar to the pattern of localisation reported by others in the postnatal rat (Hanna *et al.*, 1995; Lazowski *et al.*, 1994; Alarid *et al.*, 1992), postnatal pig (Thorp *et al.*, 1995a; Simon and Cooke, 1988), human foetus (Hill *et al.*, 1989) and chicken (Thorp *et al.*, 1995b; Ralphs *et al.*, 1990). IGF-I and IGF-II were predominantly located in hypertrophic chondrocytes throughout development, while fewer chondrocytes in the proliferative zone contained IGF-I or IGF-II. Most studies have similarly found a low number of stained cells for IGF-I in proliferating chondrocytes (Hanna *et al.*, 1995; Thorp *et al.*, 1995a; Ralphs *et al.*, 1990). However, these results contrast with the findings of Nilsson *et al* (1986) who reported the presence of IGF-I largely in the proliferative zone of the postnatal rat growth plate.

It is possible that the level of IGF-I in the proliferative zone differs between studies as a result of a change during development. In the present study, IGF-I was found to be absent in this zone from the early foetal period, but more chondrocytes contained IGF-I at later stages of foetal development.

In some chondrocyte columns, most of the cells stained intensely, whereas in other columns only a few cells were stained. Regardless of the overall number of cells positive for IGF-I, all studies report similar uneven staining between columns of proliferating chondrocytes (Thorp *et al.*, 1995a; Hanna *et al.*, 1995; Simon and Cooke, 1988; Nilsson *et al.*, 1986). However, few studies have attempted to determine its biological significance. While adjacent chondrocytes displaying IGF staining in the present study were unable to be distinguished by

their phenotype, Simon and Cooke (1988) found that chondrocytes positive for IGF-I stained red while negative chondrocytes stained blue when sections were counterstained with alcian blue-van Gieson. Although the authors did not discuss the implications of this staining pattern in relation to IGF-I action, it is tempting to speculate that IGF-I is located in those cells producing collagen and absent in those cells producing sulphated proteoglycans. Limited evidence suggests that collagen production in chondrocytes is mosaic with particular chondrocytes producing collagen while adjacent chondrocytes are negative (Aigner *et al.*, 1995; Iyama *et al.*, 1994). However further investigation is needed since IGF-I and IGF-II have both been shown to stimulate the production of collagen and non-collagen proteins such as proteoglycans in chondrocyte cell culture (Hill *et al.*, 1992a; Tyler, 1989; Luyten *et al.*, 1988; McQuillan *et al.*, 1986; Guenther *et al.*, 1982).

In contrast to the proliferative zone, an increased number of cells stained positively for IGF-I and IGF-II in the reserve cartilage zone. The proportion of positive cells for each IGF varied throughout foetal development. While at some ages a high proportion of positively stained chondrocytes were found, at other ages very low or no cells were positive. The presence of IGF-II has not been described in resting chondrocytes, but low variable levels of IGF-I (Thorp *et al.*, 1995a; Lazowski *et al.*, 1994; Ralphs *et al.*, 1990; Hansson *et al.*, 1988) or an absence of IGF-I has been found in these cells in several species (Alarid *et al.*, 1992; Simon and Cooke, 1988).

Cellular localisation of IGF-I and IGF-II in growth plate chondrocytes suggests that chondrocytes either synthesise and store IGFs or are the target for the actions of IGF. The ability of chondrocytes to synthesise IGF-I and IGF-II locally was also investigated. These results are reported in the following Chapter.

Results obtained from binding assays carried out on growth plates from different foetal ages suggest that those chondrocytes containing IGF in the proliferative and hypertrophic zones are potentially the target cells for IGF actions. Both IGF-I and IGF-II bound specifically to cells predominantly in the proliferative and hypertrophic zones. The size of the complex formed

when IGF was bound to microsomal membranes and the relative affinity of this binding in tissue sections, indicated that IGF-I and IGF-II were interacting with sites characteristic of the Type I and Type II IGF receptors, respectively (Gustafson and Rutter, 1990; Trippel *et al.*, 1988; Ballard *et al.*, 1988; Ullrich *et al.*, 1986; Beguinot *et al.*, 1985). As discussed below, an excess of IGF-I resulted in enhanced IGF-II binding which indicated that there may also be binding to IGF BPs. However, in microsomal membranes prepared from the growth plate of a late gestation foetus, IGF-II was found only to cross link to a 262 kDa species under reducing conditions which is consistent with the reported size of the Type II IGF receptor (Ballard *et al.*, 1988; Trippel *et al.*, 1988).

These results agree with *in vitro* studies which have characterised Type I and Type II IGF receptors on the membranes of chondrocytes isolated from the growth plate of several species (Bentham *et al.*, 1993; Jansen *et al.*, 1989; Trippel *et al.*, 1988; Sessions *et al.*, 1987; Schalch *et al.*, 1986; Watanabe *et al.*, 1985; Ashton and Soul, 1984; Trippel *et al.*, 1983). Although binding of IGF-I and IGF-II was higher in the proliferative and hypertrophic zones, it appears that chondrocytes located in all zones of the growth plate, except in the lower most layer of the hypertrophic zone, are capable of producing Type I and Type II IGF receptors (Wang *et al.*, 1995). Few studies however have defined which zones in the growth plate have the highest capacity to bind IGF-I and IGF-II. Makower *et al.* (1989) found that IGF-I binding in fractionated chondrocytes isolated from rat rib growth plates was two times higher in the proliferative zone, compared to the reserve cartilage or hypertrophic zones. In the present study, binding to cells was variable between zones and across foetal development. IGF-I and IGF-II binding to cells was higher in the proliferative and hypertrophic zones early in the foetal period. Much lower binding was observed in chondrocytes from the reserve cartilage zone at all stages of gestation.

The majority of matrix biosynthesis takes place in chondrocytes located in the lower proliferative, maturing and upper hypertrophic zones in connection with chondrocyte differentiation (Poole, 1991). Therefore, the pattern of localisation found for IGF-I, IGF-II

and IGF receptors in the present study suggests a role for IGF-I and IGF-II in protein synthesis.

In vitro studies have shown that IGF-I and IGF-II are able to stimulate total protein production in chondrocytes and also individually stimulate collagen, proteoglycan and non-matrix protein synthesis (Sah *et al.*, 1994; Trippel *et al.*, 1993; Hill *et al.*, 1992a; Ohlsson *et al.*, 1992a; Hill and Han, 1991; Demarquay *et al.*, 1990; Tyler, 1989; McQuillan *et al.*, 1986; Guenther *et al.*, 1982; Hill, 1979). Effects on protein synthesis observed *in vitro* are likely to represent a more general stimulatory effect on the kinetics of chondrocytes residing in the hypertrophic zone rather than an increased capacity for a particular cell to produce protein. Hunziker *et al.* (1994) have demonstrated that IGF-I treatment *in vivo* increases hypertrophic cell volume, cell height, matrix production per unit of time and the duration of time chondrocytes spend in the hypertrophic zone, but has no effect on matrix volume per cell in hypophysectomised rats.

In the same study, similar effects for IGF-I were observed on chondrocytes from the proliferative and reserve cartilage zones suggesting IGF-I is able to influence chondrocytes at all stages of differentiation (Hunziker *et al.*, 1994). However, in the present study results suggest that direct actions of IGF-I and IGF-II in the proliferative and reserve cartilage zones of foetal growth plates is restricted by very low levels of either IGF or IGF receptors. Therefore the stimulatory effects reported for IGF-I and IGF-II on cell proliferation *in vitro* (Schiltz *et al.*, 1993; Maor *et al.*, 1993a; Maor *et al.*, 1993b; Hill and Logan, 1992b; Rosselot *et al.*, 1992; Ohlsson *et al.*, 1992a; Vetter *et al.*, 1986; Hill, 1979) may be mediated by some other factor. In support of this it has been shown that the stimulation of proliferation by IGF-I *in vitro* is preceded by a lag period indicating that synthesis of other components is required before cells become responsive to IGF-I (Böhme *et al.*, 1992).

A possible mediator of IGF-I effects on proliferating chondrocytes is basic fibroblast growth factor (bFGF) which is a much more potent mitogen in chondrocyte cell culture than IGF-I or IGF-II (Trippel *et al.*, 1993; Hill and Logan, 1992b; Hill and Han, 1991). bFGF is highly expressed in the growth plate, has been shown to act synergistically with IGF-I and its

expression in costal cartilage appears to be under the control of growth hormone, a potent stimulator of bone growth (Jingushi *et al.*, 1995; Izumi *et al.*, 1995; Trippel *et al.*, 1993).

The ability for IGF-I and IGF-II to stimulate replication of chondrocytes from the proliferative zone appears to be developmentally regulated. Trippel *et al.* (1993) observed that chondrocytes from six day old rats are mitotically more responsive to bFGF than to IGF-I but more responsive to IGF-I when they are derived from twenty eight day old rats. Similarly, Vetter *et al.* (1986) observed that IGF-I was less effective at stimulating clonal growth in foetal chondrocytes than in adult chondrocytes. From these results it seems likely that direct effects of IGF-I in the proliferative zone are more limited in the foetal period compared to the postnatal period. This may explain the low level of IGF present in proliferating chondrocytes in the present study despite the presence of IGF binding sites.

The actions of IGF-I and IGF-II are modulated by IGF binding proteins (Jones and Clemmons, 1995). Of the six known IGF BPs, five are believed to be located in chondrocytes from various species (Sunic *et al.*, 1995; Doré *et al.*, 1994; Bhaumick, 1993; Olney *et al.*, 1993; Froger-Gaillard *et al.*, 1989). However their localisation in the growth plate has not been well defined. In the present study IGF BP-2, IGF BP-3, IGF BP-4 and IGF BP-5 were all co-localised in a similar pattern to IGF-I and IGF-II. This contrasts with findings in the human foetus where an absence of IGF BP-3 and -4 in the growth plate has been reported (Braulke *et al.*, 1996). The presence of more than one IGF BP at a particular location suggests a complex control system for IGF-I and IGF-II exists in the growth plate. Particular IGF BPs have been shown to be present in different forms, have differential affinities for IGF-I and IGF-II and are stimulatory or inhibitory to IGF-I and IGF-II depending on the particular tissue and stage in development (Jones and Clemmons, 1995; Bach *et al.*, 1993; Drop *et al.*, 1992). Although few studies have investigated the specific actions of IGF BPs in cartilage, IGF BP-4 has been shown to inhibit the ability of IGF-I to stimulate chondrocyte cell replication (Schiltz *et al.*, 1993).

The presence of IGF BPs in the growth plate was also indicated by a paradoxical increase in IGF-II binding when IGF-II was competed with an excess of IGF-I. This phenomenon has also been observed in a variety of other tissues (Conover, 1991; McCusker *et al.*, 1990; Barenton *et al.*, 1988; McCusker and Clemmons, 1988). It is believed to be due to a complex interaction between IGF-I, IGF-II, cell associated IGF BPs, Type I and Type II IGF receptors and IGF BPs not associated with cell membranes (McCusker *et al.*, 1990). It is predicted that cell associated IGF BPs dissociate from the membrane with an excess of competing ligand. Undissociated IGF BPs have a higher affinity for IGF than when they are membrane bound. Therefore labelled IGF-II preferentially binds to the released IGF BPs in preference to both IGF receptors and cell associated IGF BPs (Conover, 1992; McCusker *et al.*, 1990).

Although no evidence of IGF BPs was found by affinity cross linking IGF-I or IGF-II to isolated microsomal membranes, staining for all IGF BPs was found exclusively over cells in the growth plate. Since membrane associated IGF BPs are not integral to the membrane (Jones and Clemmons, 1995), it is likely that they are lost during the extraction procedure and are therefore not observed as cross linked products to IGF-I or IGF-II.

Apart from specific cellular binding, additional binding of IGF-I and IGF-II was observed over cartilage matrix. Although this was not characterised further, it is likely to be due to binding of IGF to extracellular matrix components and/or IGF BPs. Evidence suggests that IGF-I, IGF-II and IGF BPs are all capable of binding to proteins in the extracellular matrix (Jones and Clemmons, 1995; Hodgkinson *et al.*, 1995a; Hodgkinson *et al.*, 1995b; Jones *et al.*, 1993). Furthermore, IGF-I has been localised specifically to collagen within bone matrix (Slater *et al.*, 1994). It is therefore a little surprising that this and other studies have failed to locate IGF-I, IGF-II or IGF BPs in the extracellular matrix by immunohistochemistry (Middleton and Tyler, 1992; Hansson *et al.*, 1988; Nilsson *et al.*, 1986). A failure to detect particular IGF or IGF BPs by immunohistochemistry could be due to the antibody binding sites being masked by IGF binding to IGF BPs and matrix components or IGF BPs binding to IGF and matrix components. Further studies are required to confirm the presence of IGF components in cartilage extracellular matrix.

At no stage in foetal sheep bone development was there any evidence of local production of IGF BP-2, IGF BP-4 or IGF BP-5. Similarly, other studies have failed to find any significant or widespread localisation of mRNA for IGF BPs 1 to 6 in rat and mouse growth plates from the prenatal period (Wang *et al.*, 1995; Cerro *et al.*, 1993). Wang *et al.* (1995) found only very low levels of IGF BP-5 and IGF BP-6 mRNA in the reserve cartilage zone of growth plate. This contrasts with *in vitro* cell culture studies which have reported the presence of many different IGF BPs in serum free media in chondrocytes cultured from articular, limb bud and growth plate cartilage (Sunic *et al.*, 1995; Bhaumick, 1993; Froger-Gaillard *et al.*, 1989). It is unclear why *in vitro* and *in vivo* studies differ. One possibility is that in those studies utilising Western ligand blotting techniques (Sunic *et al.*, 1995; Froger-Gaillard *et al.*, 1989), binding with IGF-I or IGF-II represents binding to extracellular matrix proteins and not the reported IGF BPs However, more recently at least two of the bands believed to be IGF BPs were identified as IGF BP-2 and IGF BP-3 by immunoblotting (Olney *et al.*, 1993; Bhaumick, 1993). Formal identification of the other bands believed to be IGF BPs has yet to be carried out. Chondrocytes may be able to produce IGF BPs *in vitro* but not *in vivo* because the necessary controls to inhibit expression are lacking *in vitro*. Further investigation is required to test this possibility.

Given the remarkable similarity of IGF-I and IGF-II localisation found in the present study, it is not surprising that an absence of either IGF-I or IGF-II during prenatal development results in a similar phenotype (Liu *et al.*, 1993; Baker *et al.*, 1993; Powell-Braxton *et al.*, 1993; De Chiara *et al.*, 1990) or that the actions of IGF-I and IGF-II on chondrocytes *in vitro* are similar (Hill *et al.*, 1992a; Vetter *et al.*, 1986). These results along with other evidence has led to the concept that the actions of IGF-I and IGF-II are both exerted largely through the Type I IGF receptor (Jones and Clemmons, 1995; Schlessinger *et al.*, 1995; Lau *et al.*, 1994; Liu *et al.*, 1993; Adashi *et al.*, 1990; Furlanetto *et al.*, 1987). The relative affinities of IGF-I and IGF-II for the Type I IGF receptor (Jones and Clemmons, 1995; Germain-Lee *et al.*, 1992; Steele-Perkins *et al.*, 1988) may explain the greater potency of IGF-I when compared to IGF-II at equimolar concentrations on chondrocyte growth characteristics *in vitro* and *in vivo* (Luyten *et*

al., 1988; Schoenle *et al.*, 1985). However, the biological significance of IGF-I and IGF-II co-localisation is unclear. Koea *et al.* (1992) propose that circulating IGF-II is a modulator of the anabolic actions of IGF-I in sheep. IGF-II administration blocked the protein anabolic effects of IGF-I when normal circulating IGF-I levels were maintained (Koea *et al.*, 1992). Further characterisation of the interaction between IGF-I, IGF-II and IGF BPs in the growth plate is required.

4.4.2 Localisation and Ontogeny of IGF-I, IGF-II and IGF BP-2, -3, -4 and -5 in Bone

As was found in the growth plate, IGF-I, IGF-II and IGF BP-2, -3, -4 and -5 displayed a similar pattern of localisation. They were distributed over cells from the bone cell lineage throughout foetal development. In particular, osteoblasts displayed the highest relative staining for IGFs and IGF BPs at all locations within bone. In addition to cellular localisation, IGFs and IGF BPs were detected in bone matrix at all locations but not in cartilage remnants adjacent to bone matrix in the metaphysis.

A similar distribution of IGF-I and IGF-II in bone cells has been reported in the developing chick embryo, human foetus and postnatal rat (Braulke *et al.*, 1996; Lazowski *et al.*, 1994; Ralphs *et al.*, 1990; Hill *et al.*, 1989). In contrast to the present study no IGF was detectable in bone matrix in these studies. However, Slater *et al.* (1994) recently demonstrated the presence of IGF-I and IGF-II specifically in bone matrix associated with collagen and Hauschka *et al.* (1986) has extracted growth factors believed to be IGFs from bone matrix. Bone matrix is believed to be a storage site for many growth factors (Martin and Sank, 1991), and accounts for the high content of IGF found in bone. IGF-I and IGF-II have both been extracted from whole bone, with levels of IGF-II the highest of any growth factor in this tissue (Frolik *et al.*, 1988; Mohan *et al.*, 1986; Hauschka *et al.*, 1986) and are produced by bone cells cultured in serum free media (Birnbaum and Bowsher, 1993; McCarthy *et al.*, 1992; Mohan *et al.*, 1990; Canalis *et al.*, 1988; Mohan *et al.*, 1988a).

This pattern of localisation is consistent with numerous *in vitro* studies that suggest IGF-I and IGF-II are modest stimulators of osteoprogenitor cell replication and potent stimulators of matrix synthesis and related processes such as glucose and amino acid transport (Schmid, 1993; Raisz *et al.*, 1993; Canalis *et al.*, 1991; Pfeilschifter *et al.*, 1990; McCarthy *et al.*, 1989a; Hock *et al.*, 1988). Additionally, IGF-I and IGF-II have been shown to stimulate osteoclast activity by supporting the generation and activation of osteoclasts in bone (Hill *et al.*, 1995; Mochizuki *et al.*, 1992; Slootweg *et al.*, 1992).

These effects have been confirmed *in vivo*, where they manifest as a net increase in bone protein synthesis (Martínez *et al.*, 1994), periosteal and/or cortical bone formation (Tobias *et al.*, 1992; Machwate *et al.*, 1994; Spencer *et al.*, 1991) and bone turnover (Ebeling *et al.*, 1993). The *in vitro* effects of IGF-II on bone have yet to be confirmed *in vivo*. However, one study found an increase in plasma osteocalcin concentrations in newborn lambs treated with IGF-II (Coxam *et al.*, 1992). Plasma osteocalcin is a marker for osteoblast activity which correlates positively with bone growth (Pastoureau *et al.*, 1991).

From results obtained *in vivo*, it has been hypothesised that IGF-I and IGF-II are major factors involved in coupling bone formation to bone resorption. The presence of IGF in bone matrix found in the present study supports a model proposed by Mohan and Baylink (1991a and b) whereby IGF is able to influence bone remodelling. Local or systemic IGF is incorporated either into newly forming bone matrix (where it can be later released during bone resorption) or into extracellular fluid. IGF thereby has immediate or delayed actions on bone forming or osteoclast cells which effectively couples bone formation processes with bone absorption (Panagakos, 1993; Mohan and Baylink, 1991a and b).

The ability of IGFs to regulate bone turnover and cause a net anabolic effect on bone is determined by a host of tissue and cell specific interactions. These include interactions among other growth factors, systemic hormones, extracellular matrix and IGF BPs (Mohan and Baylink, 1991a and b). The present study found IGF BP-2, -3, -4 and -5 co-localised in bone matrix and cells that also contained IGF-I and IGF-II. In the bone cell lineage, osteoblasts

located on matrix surfaces and actively secreting bone matrix tended to show more prominent staining for IGF BPs. It therefore appears that many IGF BPs are involved in modifying IGF actions in osteoblasts, suggesting a complex system of interaction in these cells. This complexity is illustrated by differences in the behaviour of each IGF BP towards IGF. IGF BPs have been shown to exert either inhibitory or stimulatory effects on IGF actions in cultured bone cells (Rosen *et al.*, 1994; Durham *et al.*, 1994; Andress and Birnbaum, 1992; Bautista *et al.*, 1991; Scharla *et al.*, 1991; Mohan *et al.*, 1989a). Furthermore each IGF BP has a different affinity for IGF-I and IGF-II. While IGF BP-1, -3 and -4 have equal preference for IGF-I and IGF-II, IGF BP-2, -5 and -6 have a higher preference for IGF-II than IGF-I (Jones and Clemmons, 1995; Rosen *et al.*, 1994)

Various forms of all six IGF BPs are either produced by or detected in serum free media of a range of bone cell lines (Birnbaum and Wiren, 1994; Okazaki *et al.*, 1994; Hassager *et al.*, 1992; Tørring *et al.*, 1991; Mohan and Baylink, 1991a and b). One study found differential expression and secretion of IGF BPs during rat osteoblast development *in vitro*, with maximum levels of IGF BP-2 and -5 mRNA in proliferating pre-osteoblasts, while mature osteoblasts showed maximum expression of IGF BP-3, -4 and -6 (Birnbaum and Wiren, 1994). This pattern of expression was not translated in the present study into differential localisation of IGF BPs. All IGF BPs were predominantly located in mature osteoblasts, with low variable levels of staining in proliferating pre-osteoblasts within the periosteum and bone cavities. The local expression of IGF BPs was not analysed in bone cells.

Several lines of evidence suggest an important role for IGF-I at the boundary between the epiphysis (epiphyseal growth plate) and metaphysis. This area is characterised by angiogenesis, cartilage degradation and bone formation (Baron *et al.*, 1994; Caplan and Pechak, 1987; Howlett *et al.*, 1984; Howlett, 1980). Staining for IGF and IGF BPs was highly concentrated in this zone. While osteoblasts and bone matrix were clearly positive for IGFs and IGF BPs, it was difficult to determine if endothelial cells or osteoclasts located in this area contained these components. As described above, much evidence supports a role for IGF-I and IGF-II in coupling bone formation with bone degradation. However, IGF-I also

appear to play a role in linking the actions of osteoclasts, endothelial cells of capillaries and osteoblasts in this area (Formigli *et al.*, 1995; Fiorelli *et al.*, 1994).

It has recently been shown that IGF-I is a major chemotactic stimulator of pre-osteoclastic cell migration mediated by bone endothelial cells (Formigli *et al.*, 1995). This is one of the steps involved in recruitment of osteoclast precursors from the circulation into extracellular tissue (Formigli *et al.*, 1995). Similarly, IGF-I induces a dose-dependent stimulation of endothelial cell migration in cells derived from bovine bone (Fiorelli *et al.*, 1994) and bovine carotid arteries (Nakao-Hayashi *et al.*, 1992). In support of these effects, Type I IGF receptors and the production of IGF-I have been demonstrated in endothelial cells derived from bone (Fiorelli *et al.*, 1994).

It has been hypothesised by Caplan (1990) that vascularisation and myelopoiesis are the major determinants of endochondral ossification and consequently long bone growth. Therefore, it is not surprising that IGF-I and IGF-II also appear to play a role in the proliferation of endothelial cells derived from bone and other tissues, which is a key step in angiogenesis (Goad *et al.*, 1996; Roesel and Nanney, 1995; Grant *et al.*, 1993; Streeten *et al.*, 1989; Bar *et al.*, 1988). Mice with an absence of IGF-I or IGF-II display a phenotype that could be the result of impairment of angiogenesis in the metaphyseal region (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993; De Chiara *et al.*, 1990). Although bone growth is impaired, bones appear histologically normal which is consistent with a reduced rate of endothelial cell division and therefore angiogenesis. However, the roles of IGF-I and IGF-II in angiogenesis have yet to be tested in these animals.

While the detection of IGF-I in the metaphysis during foetal development in the present study supports multiple functions for IGF-I, further investigation is needed to determine the significance of IGF-II which was also distributed in this area. A role in angiogenesis for the various IGF BPs, which were also co-localised with IGF-I and IGF-II has yet to be defined.

CHAPTER 5

LOCAL PRODUCTION OF IGF-I AND IGF-II IN BONE DEVELOPMENT

5.1 INTRODUCTION

The previous chapter shows that IGF-I and IGF-II are present in skeletal tissue throughout foetal sheep development. Both IGF-I and IGF-II appear to be located intracellularly in cells from the cartilage forming and bone cell lineages and extracellularly in bone probably bound to matrix and/or IGF BPs. Although IGF BP-2, -4 and -5 did not appear to be produced locally, they were present in all bone sections in a similar pattern of localisation to IGF-I and IGF-II.

The source of skeletal IGF-I and IGF-II is unclear. These peptides may be synthesised in cells where they are located or at other nearby sites within bone. The presence of IGF mRNA and protein suggests a paracrine or autocrine mode of action. Alternatively, IGF-I or IGF-II may be synthesised in extraskeletal sites and delivered to bone via the circulation. An absence of IGF mRNA and the presence of protein within bone would suggest an endocrine mode of action.

The current chapter investigates whether IGF-I and/or IGF-II can potentially act in a paracrine or autocrine way within bone during the foetal period. The cellular location of IGF-I mRNA and IGF-II mRNA was determined in sheep bone throughout foetal development by *in situ*

hybridisation. The particular IGF transcripts and the presence or absence of IGF mRNA were confirmed by Northern analysis.

Based on these results, the ontogeny study was extended into the postnatal period, to investigate whether a switch in local production of IGF-I and IGF-II mRNA occurs postnatally in sheep. Bones from four postnatal ages were analysed for IGF-I mRNA and IGF-II mRNA by *in situ* hybridisation and Northern hybridisation.

5.2 METHODS

5.2.1 *In Situ* Hybridisation

Sections of bone from all animals in the foetal and postnatal ontogeny studies were analysed for IGF-I and IGF-II mRNA by *in situ* hybridisation (Table 5.1). Particular ages were chosen to evenly span bone development through the foetal and neonatal periods and to include bone from the adult. IGF-I mRNA was detected using an IGF-I riboprobe transcribed from a 203 bp human IGF-I cDNA, while IGF-II mRNA was detected with an IGF-II riboprobe made from a 552 bp human IGF-II cDNA (described in Chapter 2).

To analyse all bone sections from the foetal ontogeny study, it was necessary for sections to be divided into individual *in situ* hybridisation experiments. Initial experiments were divided by foetal age, with each experiment including bone sections from all animals of a particular age. For example, sections representing the proximal and distal femur, proximal and distal tibia and the diaphysis region of the femur and tibia from all 12 animals at 140 days gestation were analysed in one experiment. Later, a comparison of IGF-I or IGF-II mRNA levels between bone sections from one animal at each age were made within the same experiment. Bone sections from the postnatal ontogeny study were analysed within an individual *in situ* hybridisation experiment.

Animal Age	*In situ* hybridisation		Northern Hybridisation	
	Number of animals analysed	Bones analysed	Number of animals analysed	Bones analysed
50 days gestation	10	right hind limb	1	right fore limb
62 days gestation	11	right hind limb	1	right fore limb
82 days gestation	12	femur proximal tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
103 days gestation	12	proximal femur distal femur proximal tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
124 days gestation	12	proximal femur diaphysis femur distal femur proximal tibia diaphysis tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
140 days gestation	12	proximal femur diaphysis femur distal femur proximal tibia diaphysis tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
2 days old	2	proximal femur distal femur proximal tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
2 weeks old	2	proximal femur distal femur proximal tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
6 months old	2	proximal femur distal femur proximal tibia distal tibia	1	proximal humerus (growth plate, diaphysis)
18 months old	2	proximal femur distal femur proximal tibia distal tibia	1	proximal humerus (growth plate, diaphysis)

Table 5.1

Summary of animals and areas of bone from the foetal and postnatal ontogeny studies analysed by *in situ* hybridisation and Northern hybridisation for IGF-I and IGF-II mRNA.

Where antisense hybridisation was higher than sense hybridisation with a particular probe it was concluded that mRNA for that probe was present. Specific hybridisation for IGF-I and IGF-II mRNA was assessed at the tissue level using X-ray film and at the cellular level using emulsion and light microscopy. At the cellular level, IGF mRNA was considered to be present where there was a higher grain density in the section probed with an antisense IGF probe compared to the same site in an adjacent section probed with a sense IGF probe. The higher the specific hybridisation, the higher the level of mRNA.

The cellular location and relative abundance of IGF-I and IGF-II mRNA was assessed in bone sections from all animals in the foetal ontogeny study. Comparisons were made within a section using an arbitrary scale for each probe from high to low which reflected the relative abundance of grains for IGF mRNA in a particular area or cell. The cell type or region of bone within each section with the highest specific grain density was denoted (++++), whereas comparatively lower grain densities were categorised as (+++), (++) or (+). Where no difference could be found between sections probed with sense and antisense probes, it was concluded that no IGF mRNA was present and was therefore denoted (–). The levels of IGF-I and IGF-II mRNA were assessed on different scales, and therefore cannot be compared directly. Results reflect differences in the relative level of either IGF-I or IGF-II mRNA between areas within bone.

5.2.2 Northern Hybridisation

After the *in situ* hybridisation analyses were complete, ovine cDNAs for IGF-I and IGF-II became available. To confirm *in situ* hybridisation results obtained with probes based on human IGF sequences, both human and ovine cDNAs were used to generate probes for Northern hybridisation. RNA samples were analysed for IGF-I mRNA using probes generated from a human cDNA (203 bp) (same probe used for *in situ* hybridisation), ovine cDNA (203 bp) (same region as the 203 bp human probe) and an ovine cDNA (264 bp) (coding for a region more 3′ to the other two probes). IGF-II mRNA was detected using probes made from a human cDNA template (552 bp) (same probe as used for *in situ* hybridisation) an ovine

cDNA template (388 bp) (encompasses the region coded for by the human probe) and an ovine cDNA template (350 bp) (coding for a non-overlapping sequence 3′ to the coding region).

Small samples of whole bone, growth plate or diaphysis were microdissected from bones from the foetal and postnatal ontogeny studies. Total RNA was extracted from these samples and analysed for IGF-I and IGF-II mRNA. Probes were sequentially hybridised to the same blot. After hybridisation, each probe was stripped from the membrane by washing in 0.1% w/v SDS at 100°C for two periods of 30 minutes (Maniatis *et al.*, 1982). The removal of probe was confirmed by exposing the membrane to X-ray film for at least 7 days.

RNA samples extracted from two regions of bone from an animal at each age of the foetal and postnatal ontogeny studies were analysed on at least two membranes, with each of the IGF probes described (Table 5.1).

5.3 RESULTS

5.3.1 *In Situ* Hybridisation

5.3.1.1 IGF-I mRNA and IGF-II mRNA in Foetal Development

The relative levels of IGF-I mRNA were significantly lower than levels of IGF-II mRNA in bones from all animals and stages of development. IGF-I mRNA was barely detectable in bone, as judged by the small difference between sense and antisense hybridisation (Figure 5.1). The overall levels of IGF-I mRNA did not change between particular bones (tibia or femur), the different ends of each bone (distal or proximal) or between bones from animals at the same age (Figure 5.1). There was also little difference in the level of IGF-I mRNA in bone across the six foetal ages (Figure 5.1).

In contrast to the low levels of mRNA observed for IGF-I, high levels of IGF-II mRNA were found throughout foetal development (Figure 5.2). Levels of IGF-II mRNA were similar

Figure 5.1

Autoradiographic images of bone sections from the foetal ontogeny study probed with either an antisense or sense human IGF-I probe in adjacent sections.
Bones from one representative animal at each age are presented
X-ray film exposure was for 2 days.
A represents antisense hybridisation
S represents sense hybridisation in adjacent sections
Liver from a 7 day old lamb, previously shown to contain IGF-I mRNA, is presented for comparison

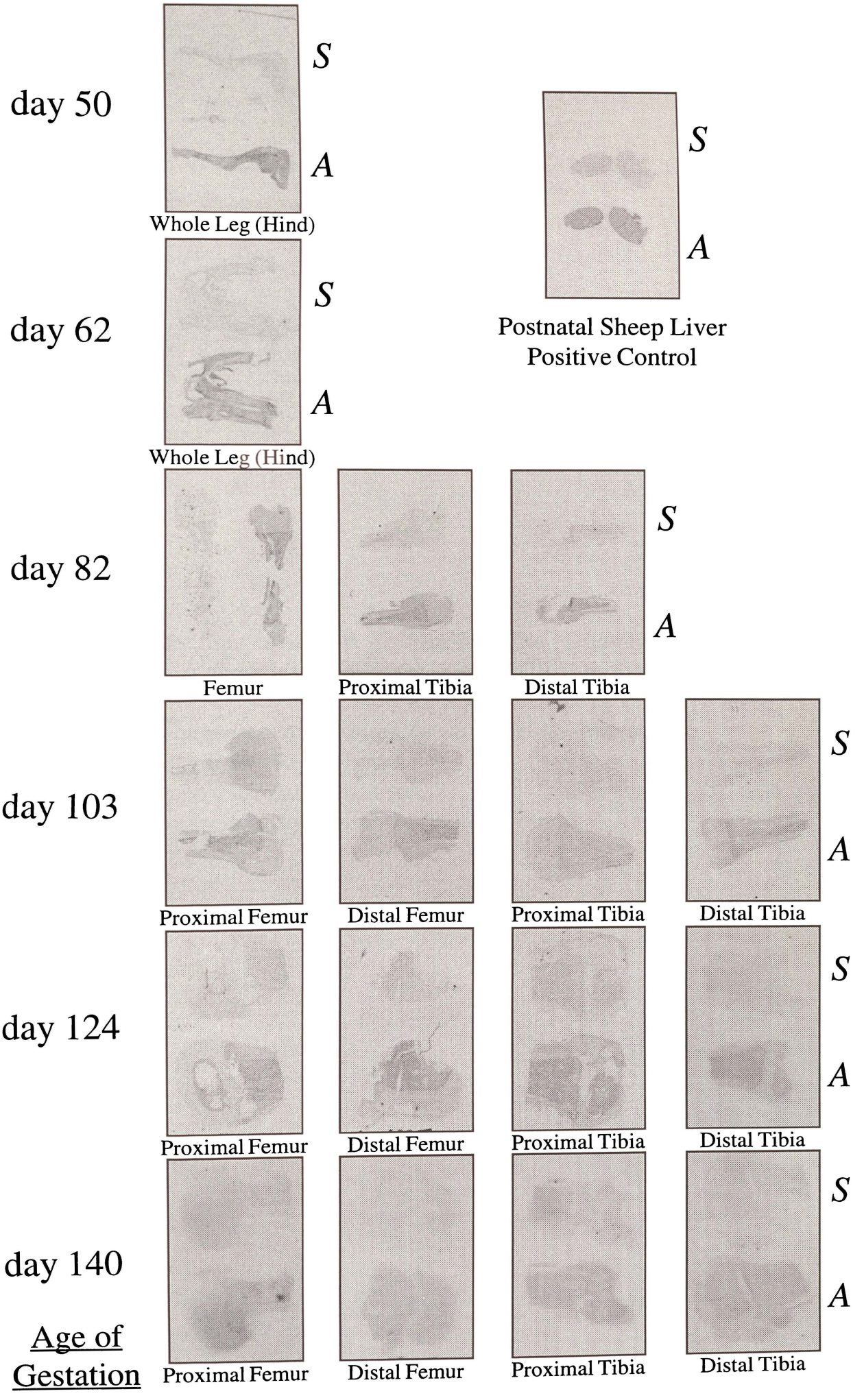
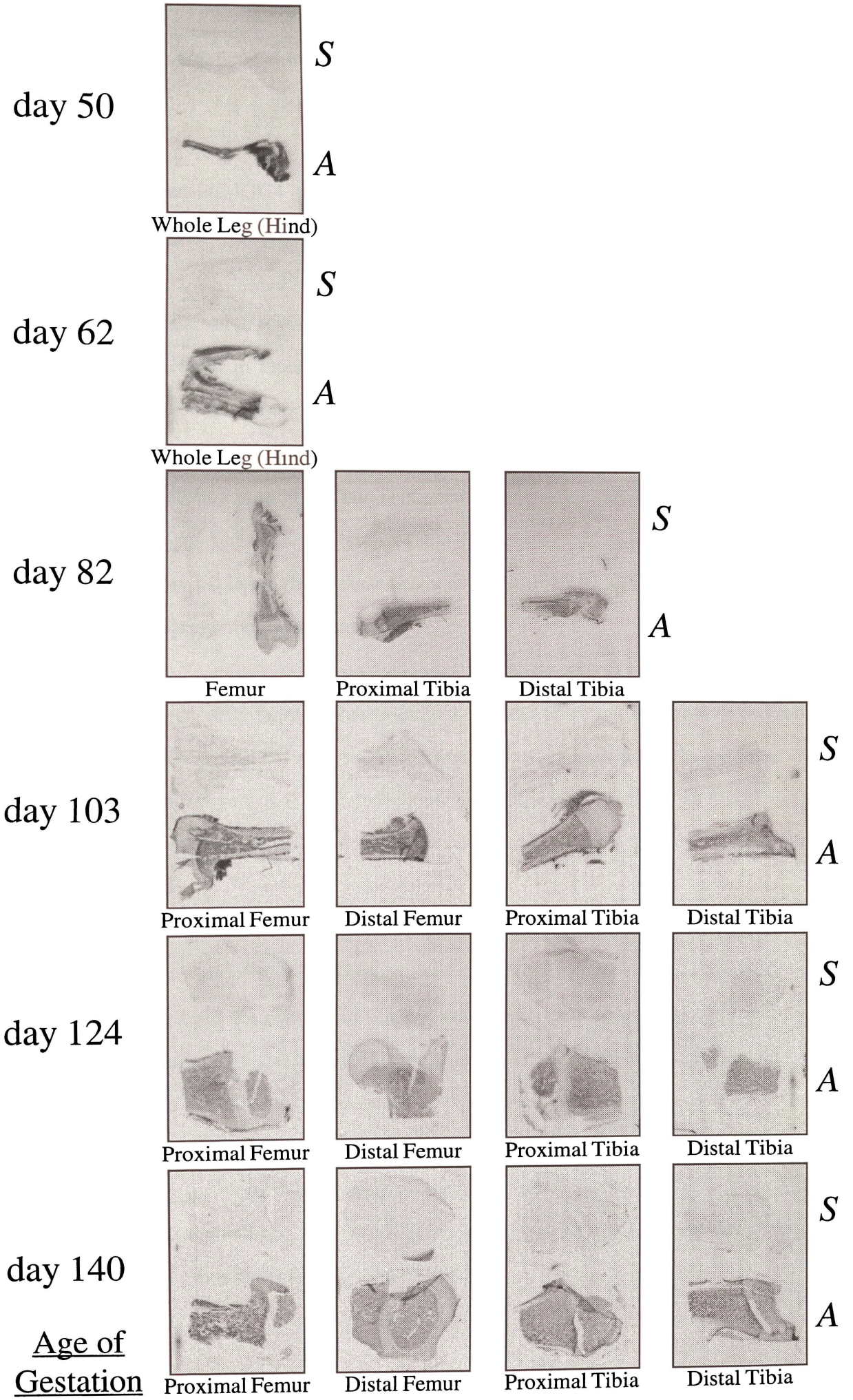

day 50
S
A
Whole Leg (Hind)
S
A
Postnatal Sheep Liver
Positive Control
day 62
S
A
Whole Leg (Hind)
day 82
S
A
Femur
Proximal Tibia
Distal Tibia
day 103
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 124
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 140
S
A
Age of Gestation
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia

Figure 5.2

Autoradiographic images of bone sections from the foetal ontogeny study probed with either an antisense or sense human IGF-II probe in adjacent sections.
Bones from one representative animal at each age are presented.
X-ray film exposure was for 30 hours.
A represents antisense hybridisation
S represents sense hybridisation in adjacent sections


day 50
S
A
Whole Leg (Hind)
day 62
S
A
Whole Leg (Hind)
day 82
S
A
Femur
Proximal Tibia
Distal Tibia
day 103
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 124
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
day 140
S
A
Proximal Femur
Distal Femur
Proximal Tibia
Distal Tibia
Age of Gestation

between bones and animals at a particular age and across all the foetal ages studied (Figure 5.2).

Particular cells expressing IGF-I and IGF-II were identified by their histological appearance and location within bone. The characteristics of these cells have been described in detail in Chapter 1.

5.3.1.1.1 *Localisation of IGF-I mRNA*

IGF-I mRNA was present in most cells and areas of bone, with the relative levels between cells showing considerable variation. Some cells displayed a high level of IGF-I expression, while other cells contained much lower or no IGF-I expression. The overall pattern of localisation found within a particular bone section was generally similar across all the bones, animals and foetal ages that were analysed (Table 5.2). Areas of cartilage degradation and active bone formation displayed the highest levels of IGF-I mRNA, although overall levels of IGF-I mRNA were barely above background.

Chondrogenesis

IGF-I mRNA levels in cells from the cartilage cell lineage were barely above background. Very low levels of IGF-I mRNA were found in chondrogenic cells residing in the inner layer of the perichondrium (Figure 5.3). Chondrogenic cells remained positive for IGF-I mRNA throughout the foetal period until 140 days gestation, when IGF-I mRNA could no longer be detected in these cells.

In the growth plate, levels of IGF-I mRNA increased as chondrocytes passed through defined stages of maturation from the reserve cartilage zone to the hypertrophic zone. Chondrocytes in the reserve cartilage zone contained extremely low levels of IGF-I at all times in foetal development. Once these cells differentiated and became part of the proliferative zone, slightly higher levels of IGF-I mRNA could be detected (Figure 5.3). A further increase in the level of IGF-I mRNA occurred when these cells matured into hypertrophic cells (Figure 5.3). Hypertrophic cells contained the highest IGF-I expression of any of the cartilage cells, but levels were still lower than those found in osteoblast cells.

IGF-I mRNA	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	+	+	+	+	++	–
reserve zone (chondrocytes)	+	+	+	+	+	–
proliferative zone (chondrocytes)	++	++	++	++	++	–
hypertrophic zone (chondrocytes)	+++	+++	+++	+++	+++	++
periosteum (osteogenic cells)	+	+	+	+	++	–
bone cavity (osteogenic cells)	+	+	+	+	++	–
osteoblasts	++++	++++	++++	++++	++++	++++
osteocytes	–	–	–	–	–	–
zone of cartilage degradation	++++	++++	++++	++++	++++	++++
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	n.d.	++++
muscle fibres	++	++	n.d.	n.d.	n.d.	n.d.

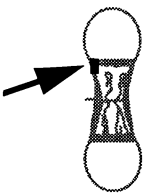
Table 5.2

Summary of the localisation and relative abundance of IGF-I mRNA in different cells and regions of bone. All bones and animals from the foetal ontogeny study were analysed.
Specific grain density was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where hybridisation was not determined; (–) indicates an absence of specific hybridisation.

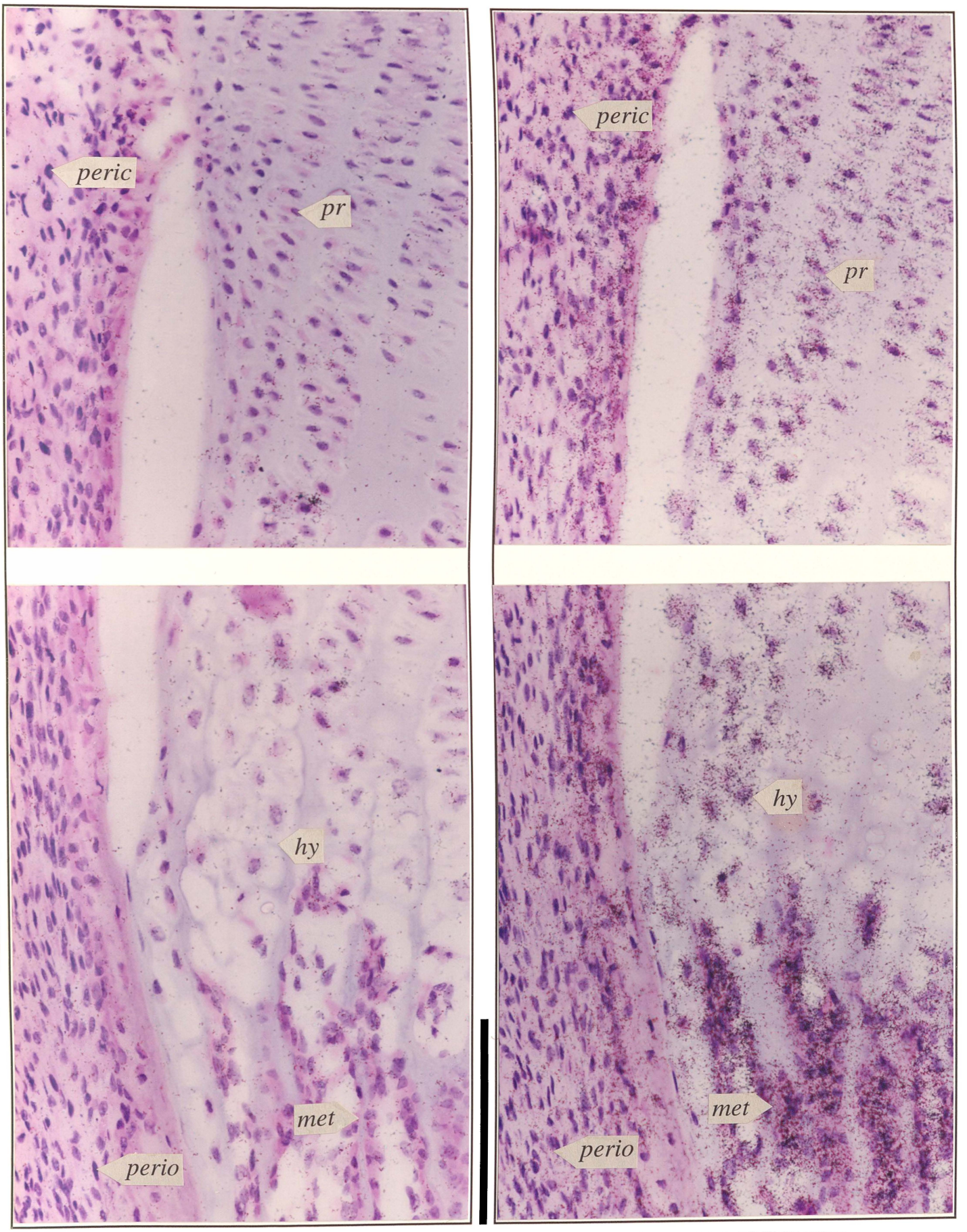
Figure 5.3 (a, b)

Microautoradiographic images of bone sections from the proximal tibia of an 82 day gestation animal from the foetal ontogeny study. Sections were probed with either a sense (a) or antisense (b) human IGF-I probe in adjacent sections.

Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)



Specific hybridisation to IGF-I mRNA is the difference between the density of black grains between antisense and sense hybridisation.
Exposure to emulsion was for 69 days
peric, perichondrium; *pr*, proliferative; *hy*, hypertrophic chondrocyte; chondrocyte; *perio*, periosteum; *met*, metaphysis
Vertical Bar = 100 μm



5.3a SENSE

5.3b ANTISENSE

Osteogenesis

As bone cells differentiated from osteogenic cells to osteocytes, the relative expression of IGF-I changed. Overall, bone cells in the earliest stages of development contained very low levels of IGF-I mRNA. As differentiation proceeded and these cells became osteoblasts, the abundance of IGF-I mRNA was greatly increased. When osteoblasts differentiated to form osteocytes IGF-I expression was down regulated. No IGF-I mRNA could be detected in osteocytes regardless of which region of bone was analysed.

At the earlier foetal ages, low levels of IGF-I mRNA were found in a distinctive layer of cells in the periosteum. Based on their location in the inner zone, these cells were presumed to be osteogenic cells (Figure 5.4). Osteogenic cells located at other sites within bone such as cavities found in the interior of bone also displayed a similar low level of IGF-I expression.

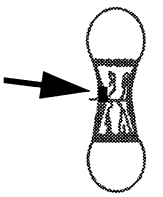
The level of IGF-I mRNA increased once osteogenic cells differentiated into osteoblasts. Osteoblasts contained higher levels of IGF-I mRNA than any other cell types found in bone, although the abundance of IGF-I mRNA in these cells was still very low compared to the levels found for IGF-II mRNA in bone. Osteoblasts expressed high levels of IGF-I mRNA regardless of where they were located or from which age the bones came from (Figure 5.4). Osteoblasts lining endosteal cavities, adjacent to the periosteum and associated with the secondary centre of ossification in the older animals all contained IGF-I mRNA (Figure 5.4). Osteoblasts located in the area of bone directly adjacent to the growth plate also expressed IGF-I mRNA. However, it was difficult to identify particular cells within this area, which contains a mixture of cells at different stages of differentiation (Figure 5.3). The abundance of IGF-I mRNA in osteoblasts did not appear to be related to the rate of bone matrix synthesis since active and resting osteoblasts (bone lining cells) contained similar levels of IGF-I mRNA (Figure 5.4).

As osteoblasts secreted and became encased in bone matrix, they differentiated into osteocytes. This coincided with down regulation of IGF-I mRNA. Osteocytes at all sites

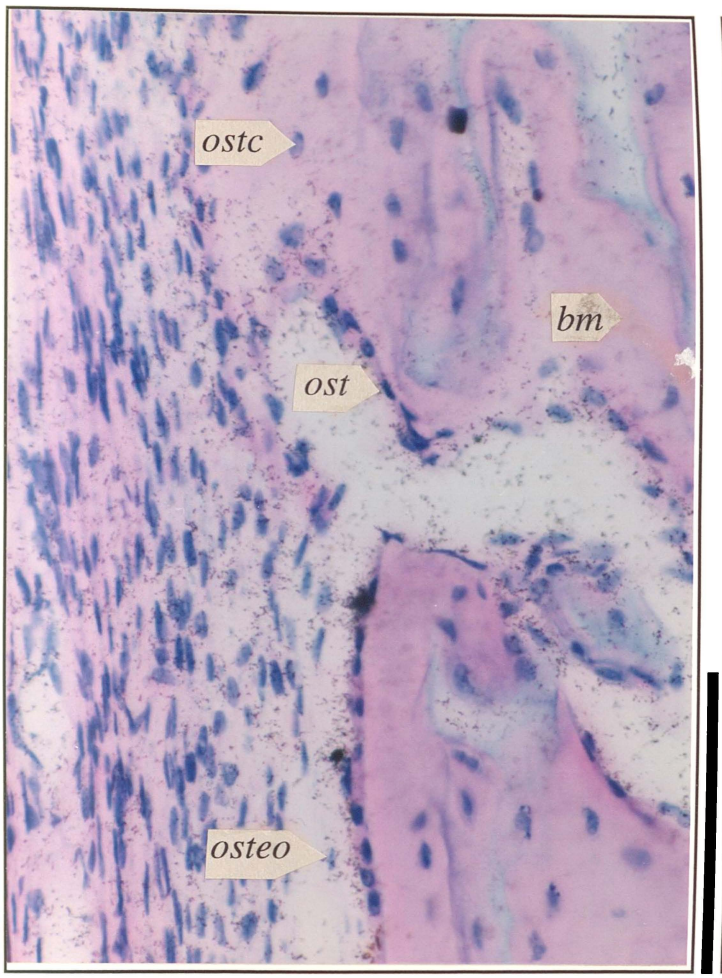
Figure 5.4 (a, b, c, d)

Microautoradiographic images of bone sections from the proximal tibia of an 82 day (a and b) and a 140 day (c and d) gestation animal from the foetal ontogeny study. Sections were probed with either a sense (a and c) or antisense (b and d) human IGF-I probe in adjacent sections.

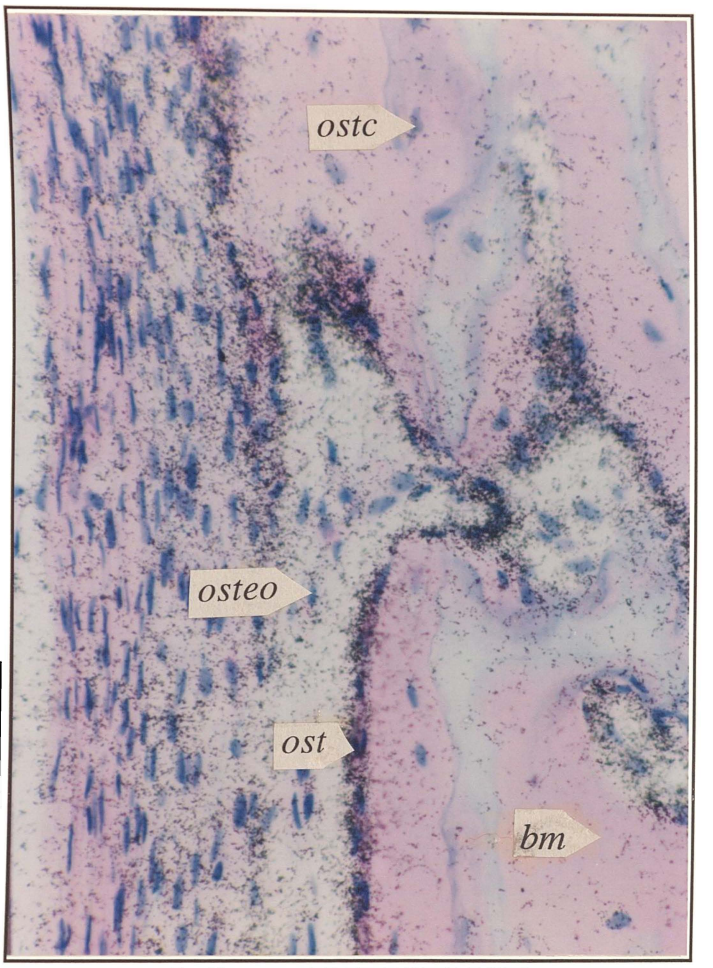
Photographs are orientated in the longitudinal plane:
(a view through the diaphysis)



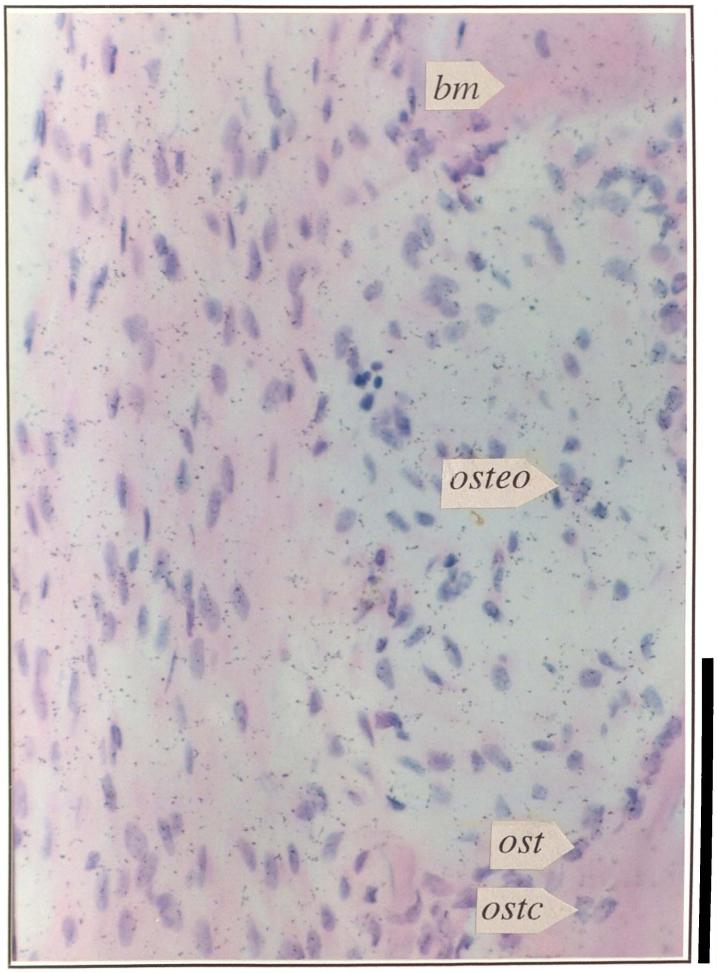
Exposure to emulsion was for 69 days (82 days gestation) or 73 days (140 days gestation)
osteo, osteogenic cell; *ost*, osteoblast; *ostc*, osteocyte; *bm*, bone matrix
Vertical Bar = 100 μm



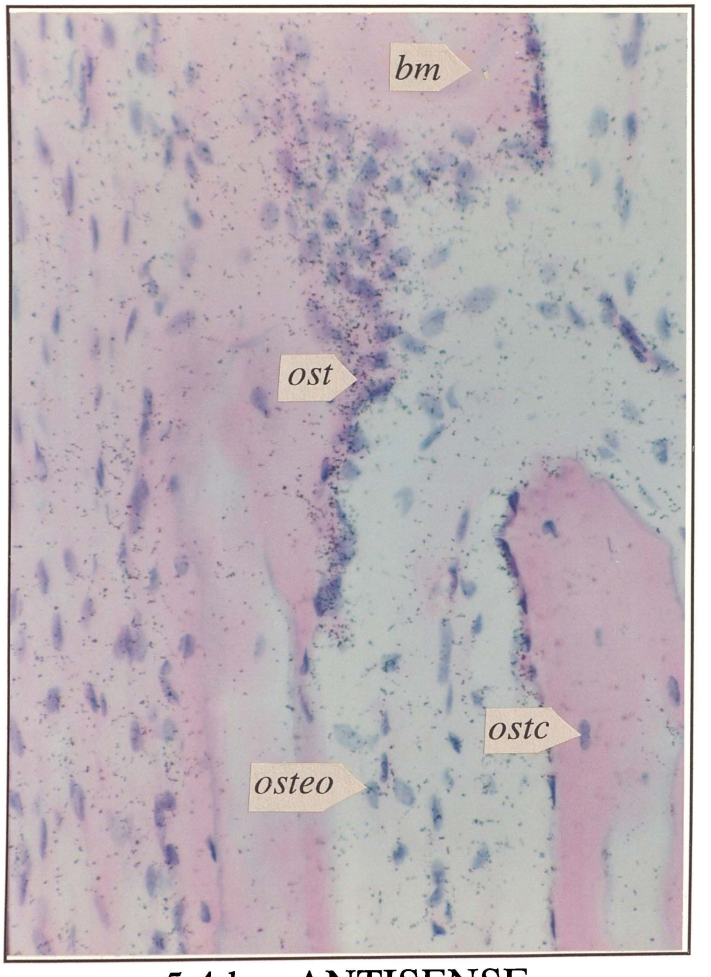
5.4a SENSE



5.4b ANTISENSE



5.4c SENSE



5.4d ANTISENSE

within bone and all stages of foetal development were negative for IGF-I mRNA (Figure 5.4). The lack of IGF-I mRNA in osteocytes was not due to an inability to detect RNA since the presence of RNA was confirmed by the detection of ribosomal RNA in these cells (Figure 5.5).

Osteoclasts

The presence or absence of IGF-I mRNA in osteoclasts could not be determined since osteoclasts could not be positively identified in these sections. The regions of bone where osteoclasts are known to reside, such as the zone of degrading cartilage, was found to contain an abundance of IGF-I mRNA. Further investigation is needed to find a marker for these cells to assess whether IGF-I mRNA found in this area was localised to osteoclasts.

5.3.1.1.2 *Localisation of IGF-II mRNA*

A high level of IGF-II expression was found in bone. IGF-II mRNA was widely distributed in many cells and areas of bone. The pattern of localisation was similar not only among bones from the same animal, but also among bones from different animals of the same age and from animals at different stages of foetal development (Table 5.3). IGF-II mRNA was detectable at all stages of chondrocyte differentiation from the reserve cartilage zone to the hypertrophic zone. IGF-II mRNA was also very high at most stages of bone cell development. There was, however, a trend for IGF-II mRNA levels to be lower in the less mature cartilage and bone cells at later stages of foetal development.

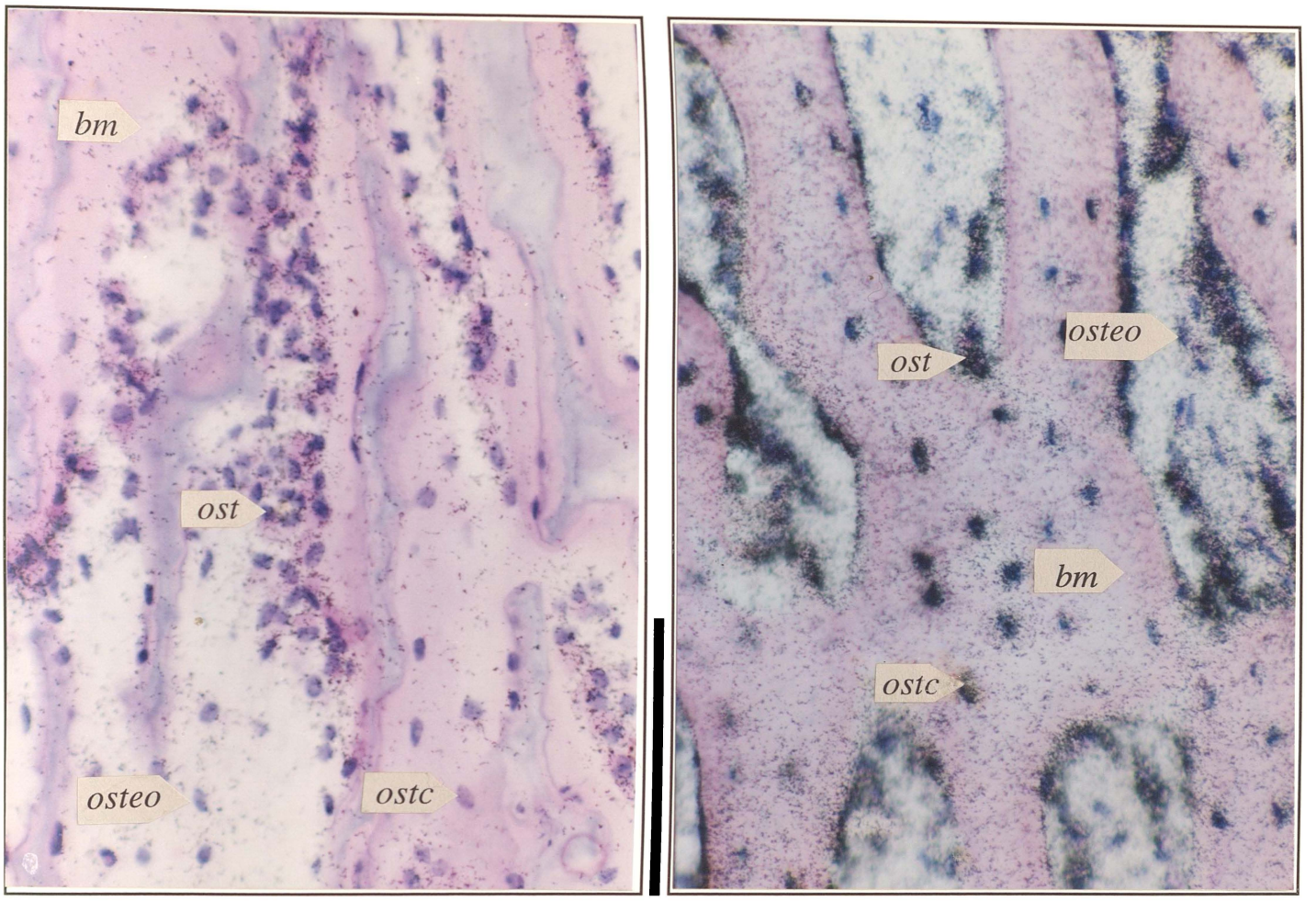
Chondrogenesis

Levels of IGF-II mRNA were very high in chondrogenic cells residing in the inner layer of the perichondrium (Figure 5.6). This was more apparent earlier in gestation, with levels of IGF-II mRNA becoming much reduced in this zone by 140 days gestation (Figure 5.6). In the reserve cartilage zone, a mixture of chondrocytes expressing, and chondrocytes not expressing, IGF-II was found (Figure 5.7). Based on their histological appearance, chondrocytes containing IGF-II mRNA could not be distinguished from adjacent chondrocytes that were not expressing IGF-II. Both expressing and non-expressing chondrocytes were evenly distributed throughout the reserve cartilage zone.

IGF-II mRNA	50 days gestation	62 days gestation	82 days gestation	103 days gestation	124 days gestation	140 days gestation
perichondrium (chondrogenic cells)	+++	+++	++++	+	+	+
reserve zone (chondrocytes)	+++	+++	+++	+	+	+
proliferative zone (chondrocytes)	+	++	++	++	++	++
hypertrophic zone (chondrocytes)	++++	+++	++++	++++	++++	++++
periosteum (osteogenic cells)	++++	++++	++++	++++	+	+
bone cavity (osteogenic cells)	++++	++++	++++	++++	++++	++++
osteoblasts	++	++	++	++	++	++
osteocytes	–	–	–	–	–	–
zone of cartilage degradation	+	++	++	–	–	–
secondary centre of ossification (osteocytes, osteoblasts, osteogenic cells)	n.d.	n.d.	n.d.	n.d.	n.d.	++++
muscle fibres	++++	++++	++++	++++	n.d.	n.d.

Table 5.3

Summary of the localisation and relative abundance of IGF-II mRNA in different cells and regions of bone. All bones and animals from the foetal ontogeny study were analysed.
Specific grain density was assessed on a scale using arbitrary units from high (++++) to low (+); (n.d.) indicates a region or cell type where hybridisation was not determined; (–) indicates an absence of specific hybridisation.

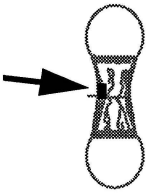


5.5a ANTISENSE 5.5b ANTISENSE

Figure 5.5 (a, b)

Microautoradiographic images of bone sections from the proximal tibia of a 50 day gestation animal from the foetal ontogeny study. Sections were probed with either an antisense human IGF-I probe (a) or an antisense ribosomal RNA probe (b) in adjacent sections.

Photographs are orientated in the longitudinal plane:
(a view through the diaphysis)

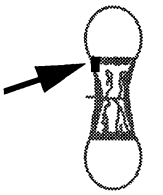


Exposure to emulsion was for 69 days (IGF-I mRNA) or 4 days (ribosomal RNA)
osteo, osteogenic cell; *ost*, osteoblast; *ostc*, osteocyte; *bm*, bone matrix
Vertical Bar = 100 μm

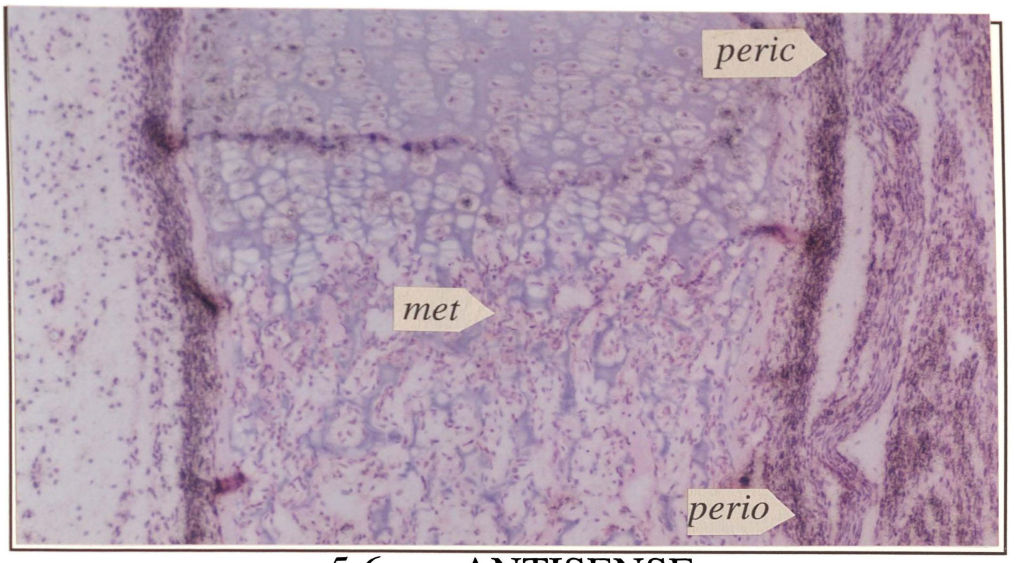
Figure 5.6 (a, b, c, d, e, f)

Microautoradiographic images of bone sections from the proximal femur of a 50 day (a, b and c) and a 140 day (d, e and f) gestation animal from the foetal ontogeny study. Sections were probed with either a sense (c and f) or antisense (a, b, d and e) human IGF-II probe in adjacent sections.

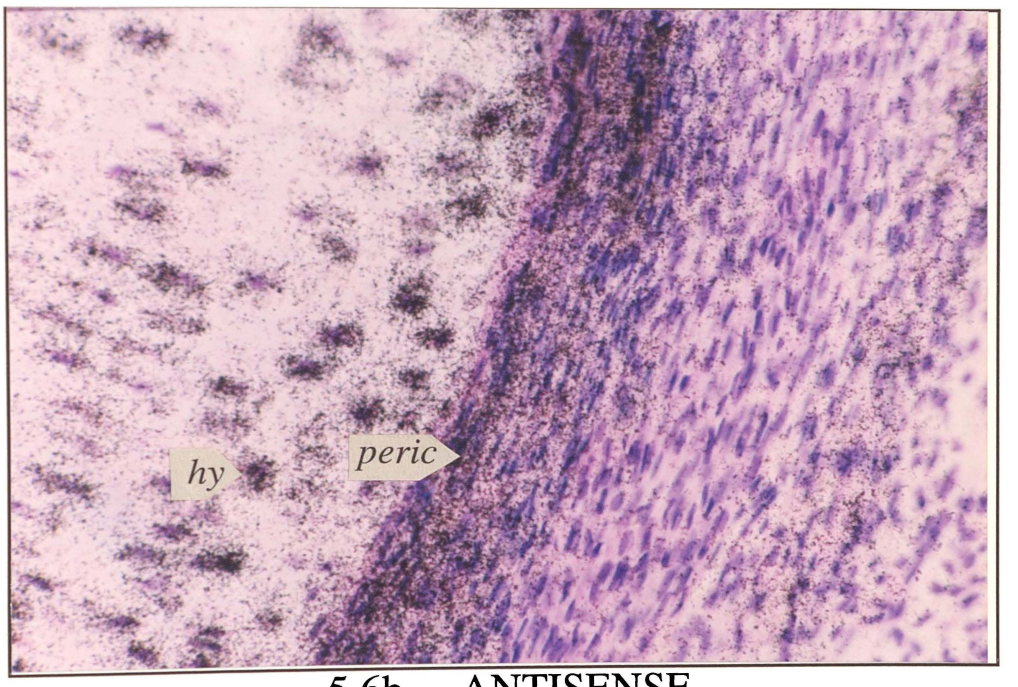
Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)



Exposure to emulsion was for 10 days (50 days gestation) or 31 days (140 days gestation)
peric, perichondrium; *gp*, growth plate; *hy*, hypertrophic chondrocyte; *pr*, proliferative chondrocyte; *perio*, periosteum; *met*, metaphysis
Vertical Bar = 100 μm



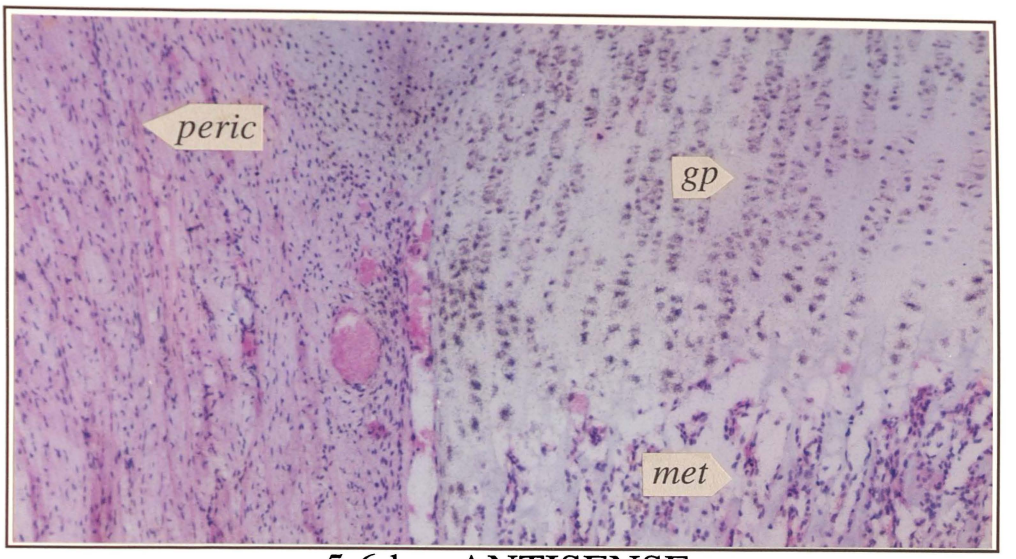
5.6a ANTISENSE



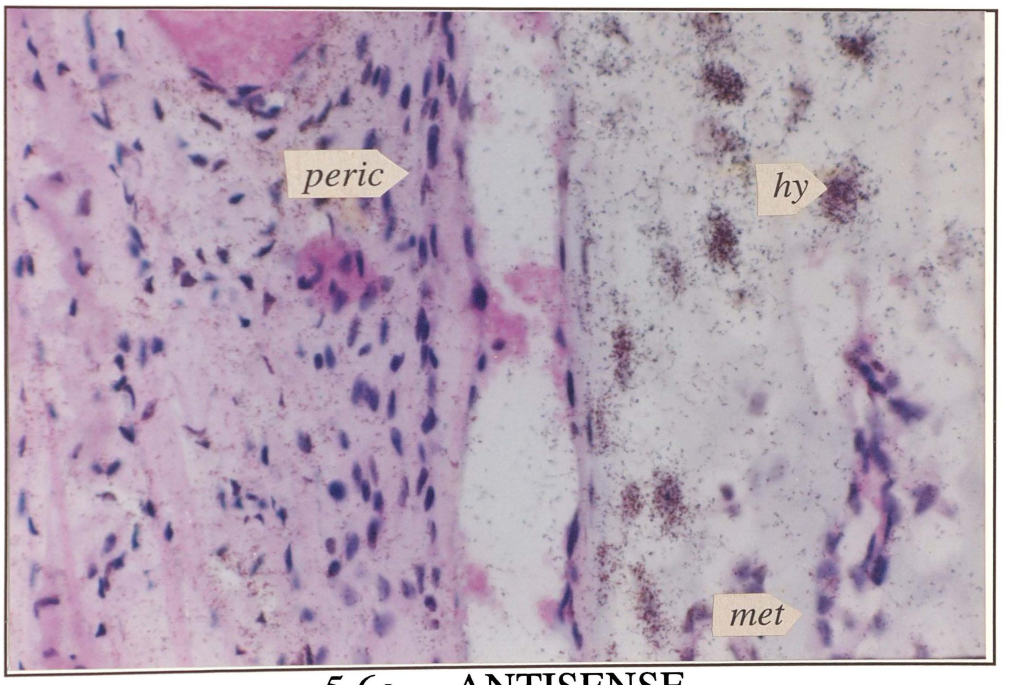
5.6b ANTISENSE



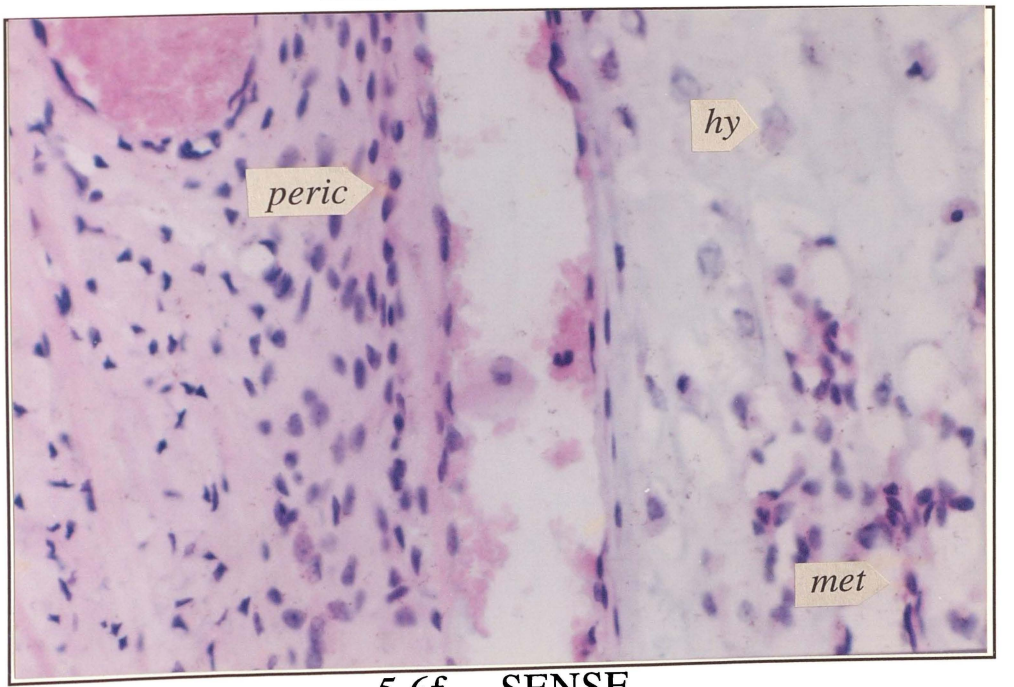
5.6c SENSE



5.6d ANTISENSE



5.6e ANTISENSE

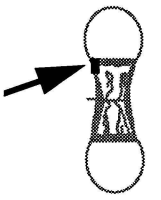


5.6f SENSE

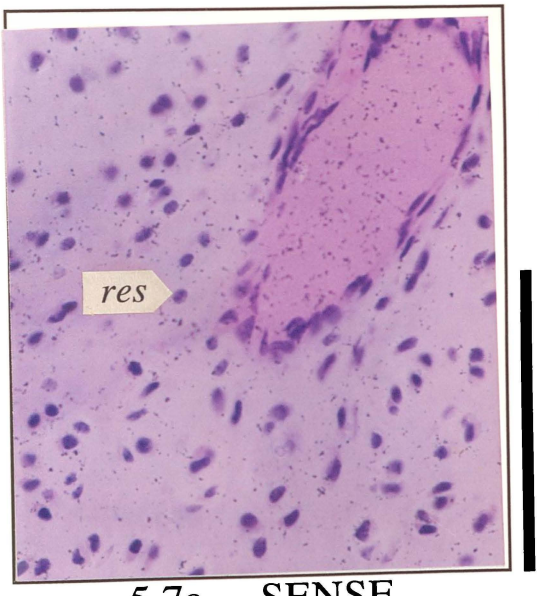
Figure 5.7 (a, b, c, d, e, f, g, h)

Microautoradiographic images of bone sections from the proximal femur of a 103 day gestation animal from the foetal ontogeny study. Sections were probed with either a sense (a, c, e and g) or antisense (b, d, f and h) human IGF-II probe in adjacent sections.

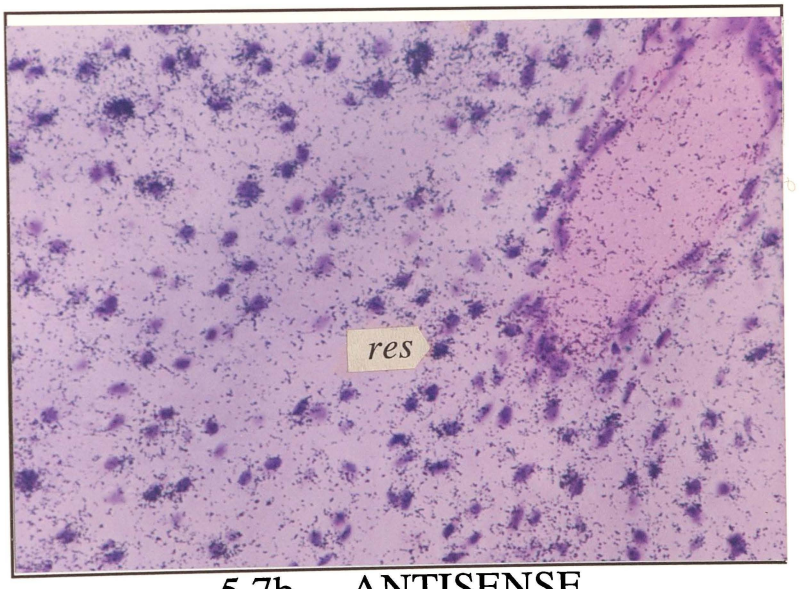
Photographs are orientated in the longitudinal plane:
(a view through the growth plate)



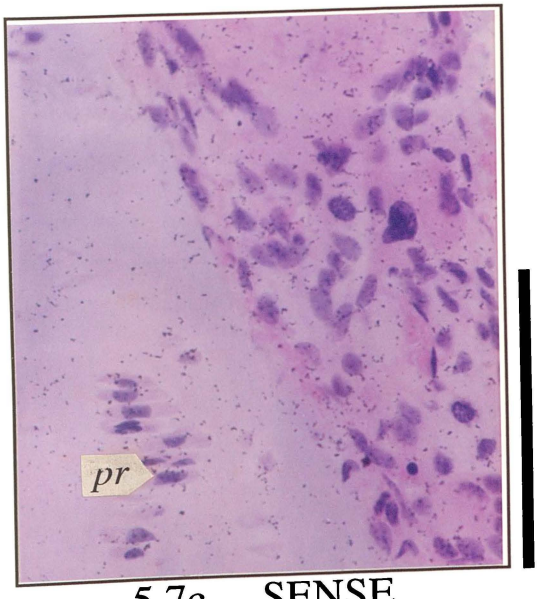
Exposure to emulsion was for 28 days
res, reserve chondrocyte; *pr*, proliferative chondrocyte; *hy*, hypertrophic chondrocyte; *met*, metaphysis
Vertical Bar = 100 µm



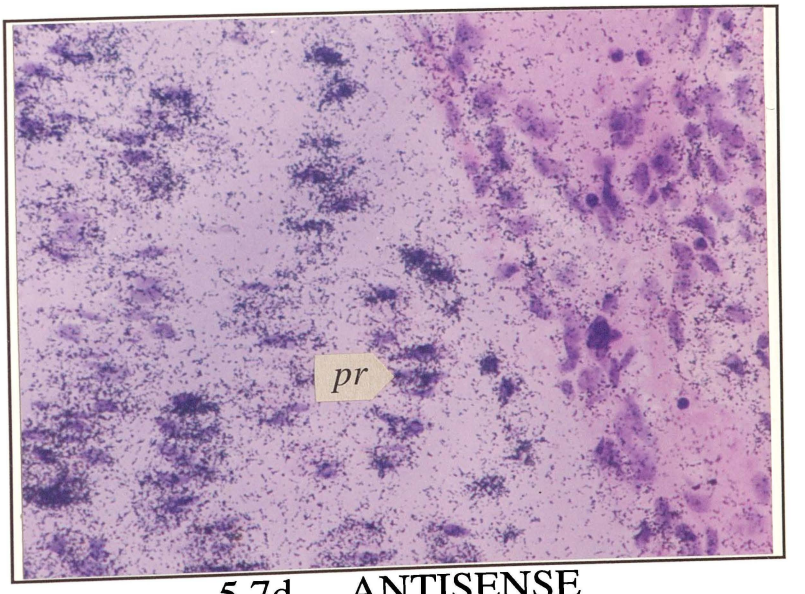
5.7a SENSE



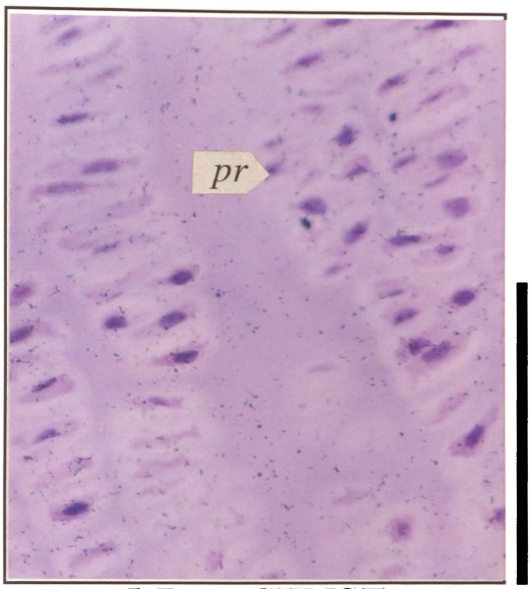
5.7b ANTISENSE



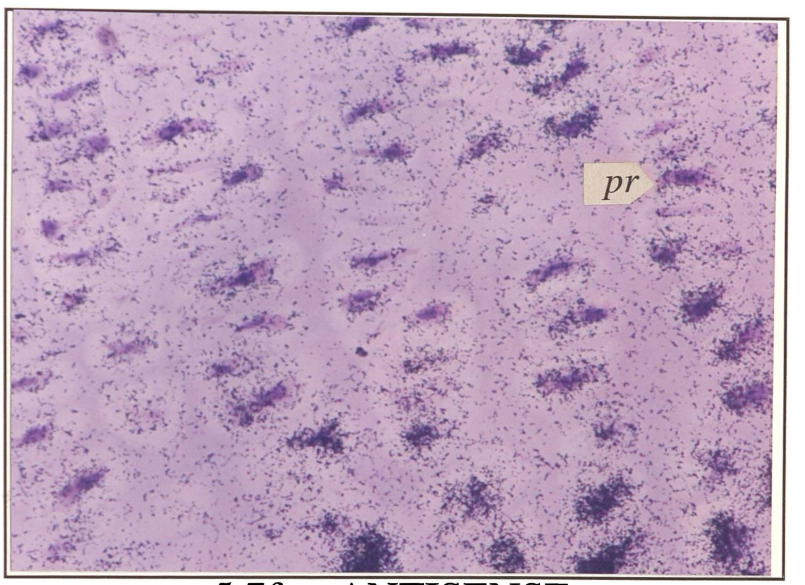
5.7c SENSE



5.7d ANTISENSE



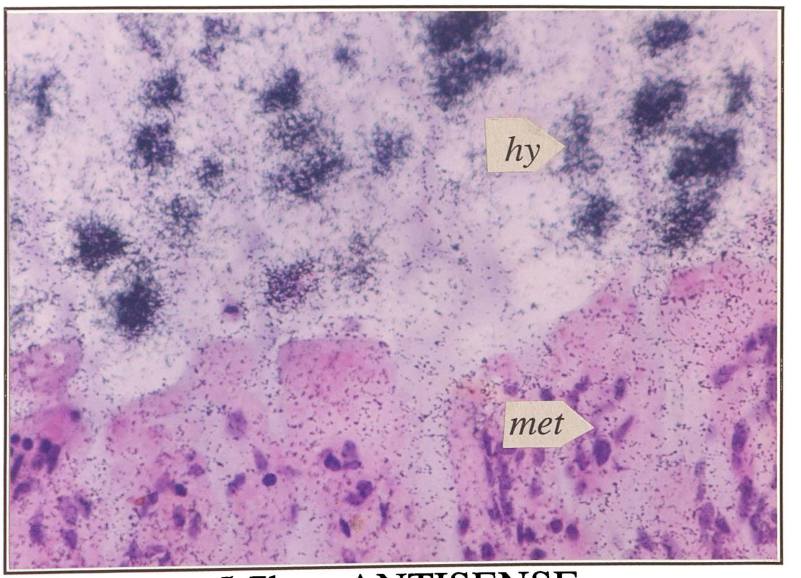
5.7e SENSE



5.7f ANTISENSE



5.7g SENSE



5.7h ANTISENSE

Once chondrocytes differentiated and became part of the proliferating zone, the levels of IGF-II mRNA in these cells declined slightly at the earlier foetal ages but increased slightly in the older foetal bones (Figure 5.7). Within the proliferating zone, there was variability in the level of expression between columns of chondrocytes. Some columns of chondrocytes contained IGF-II mRNA, whereas chondrocytes in other columns contained lower or no IGF-II mRNA (Figure 5.7).

As chondrocytes continued to mature and form hypertrophic cells, the level of IGF-II mRNA increased dramatically. Almost every hypertrophic chondrocyte was heavily labelled in this zone (Figure 5.7). Peripherally located chondrocytes adjacent to the perichondrium had a much stronger signal than those located at the centre (Figure 5.6).

Osteogenesis

Osteogenic cells in bone from all stages of foetal development, displayed a higher level of IGF-II expression than any other cell from the bone cell lineage. Almost every osteogenic cell residing in the inner layer of the periosteum (at the earlier foetal ages) and in internal bone cavities (at all ages) was intensely labelled (Figure 5.8). Osteogenic cells containing IGF-II mRNA were located within bone cavities in the metaphysis, diaphysis and marrow, where they appeared as individual cells not in contact with nearby bone matrix. Once these cells differentiated into osteoblasts and became located next to bone matrix, the level of IGF-II expression declined markedly (Figure 5.8). Although most osteoblasts still contained IGF-II mRNA, the levels were much lower than that observed for osteogenic cells (Figure 5.9). Levels of IGF-II mRNA were reduced even further as osteoblasts differentiated and became osteocytes. All osteocytes were negative for IGF-II mRNA regardless of where they were located in bone or from which bone or animal they came from (Figure 5.8 and 5.9).

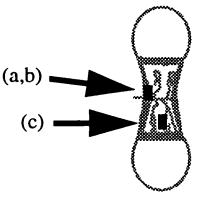
Osteoclasts

As already mentioned in the previous section, the positive identification of osteoclasts could not be made. Based on their predicted location, it was concluded that they were unlikely to be expressing IGF-II. Cells in the area of cartilage degradation, where osteoclasts are believed to be found, were negative for IGF-II mRNA. In the diaphysis, only mononucleated cells from the bone forming lineage contained IGF-II mRNA.

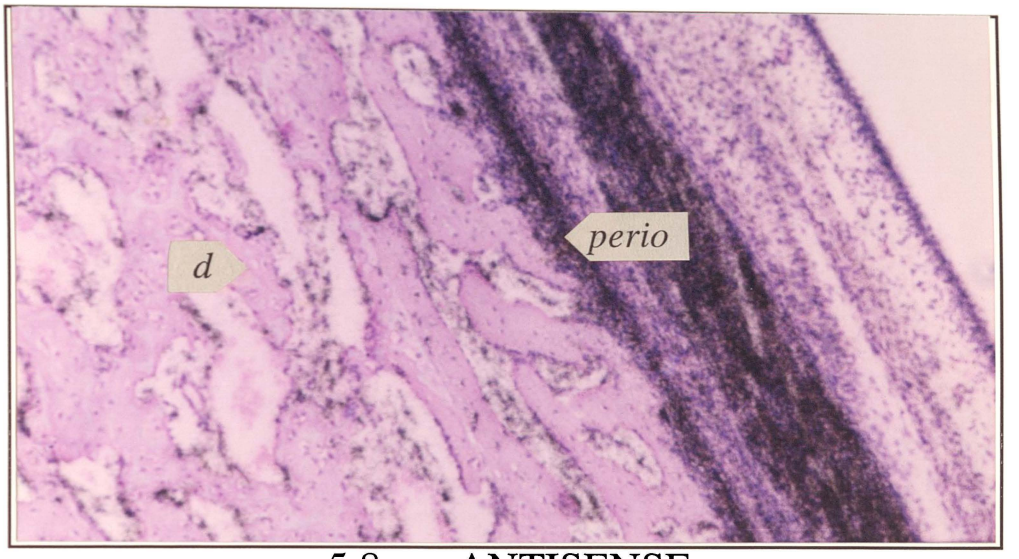
Figure 5.8 (a, b, c)

Microautoradiographic images of bone sections from the proximal femur of a 50 day gestation (a and b) and 103 day gestation (c) animal from the foetal ontogeny study. Sections were probed with either an antisense (a and c) or sense (b) human IGF-II probe in adjacent sections.

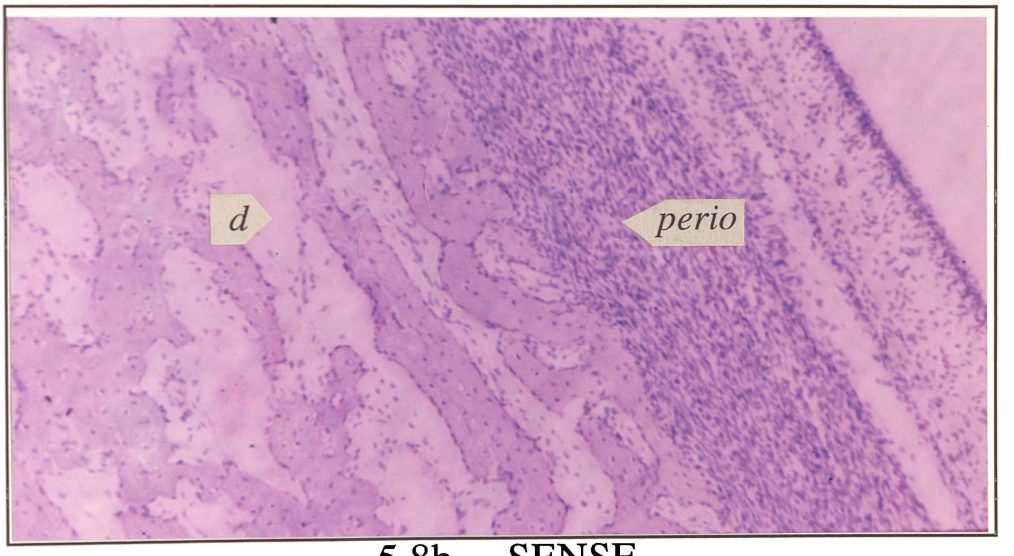
Photographs are orientated in the longitudinal plane:
(a view through the diaphysis)



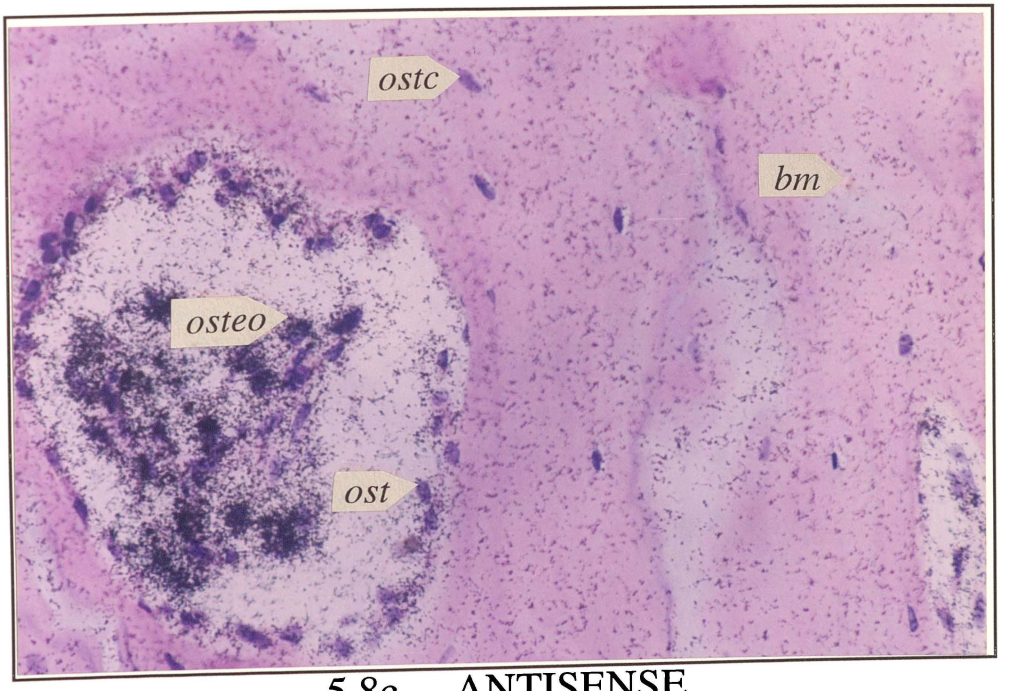
Exposure to emulsion was for 17 days (a and b) or 28 days (c)
perio, periosteum; *d*, diaphysis; *osteo*, osteogenic cell; *ost*, osteoblast; *ostc*, osteocyte; *bm*, bone matrix
Vertical Bar = 100 μm



5.8a ANTISENSE



5.8b SENSE

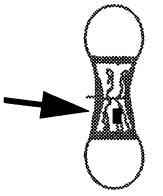


5.8c ANTISENSE

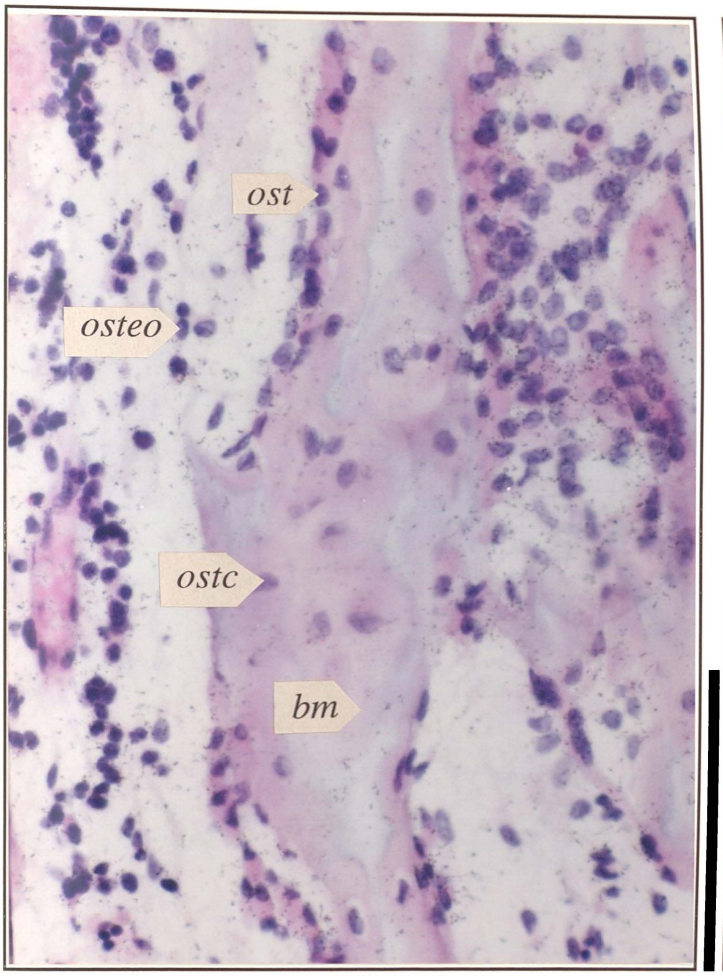
Figure 5.9 (a, b, c, d)

Microautoradiographic images of bone sections from the proximal tibia of a 103 day (a and b) and a 140 day (c and d) gestation animal from the foetal ontogeny study. Sections were probed with either a sense (a and c) or antisense (b and d) human IGF-II probe in adjacent sections.

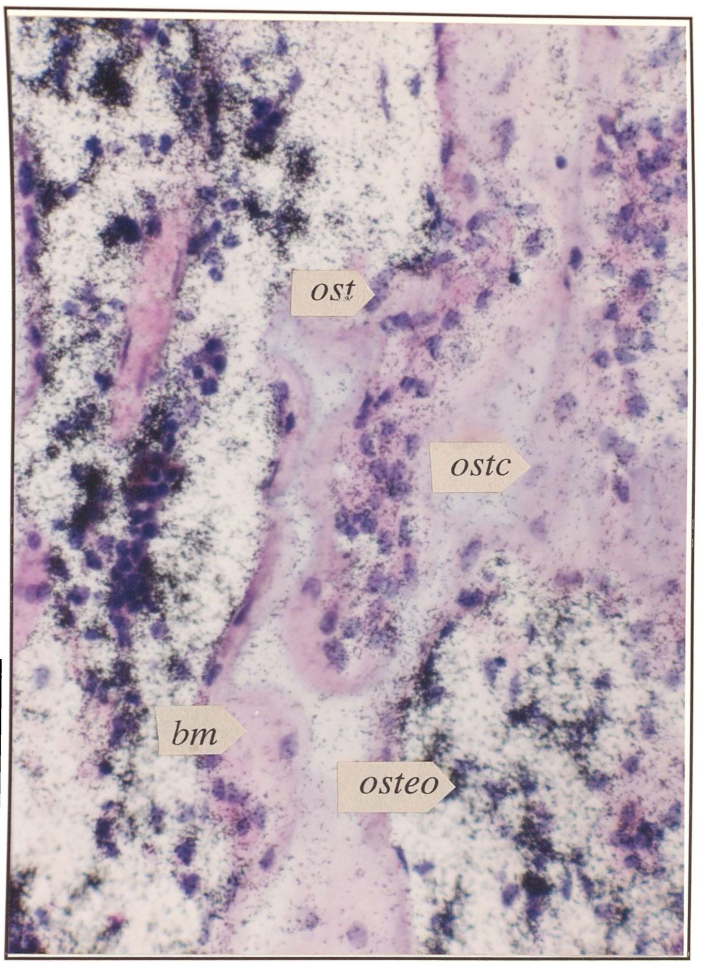
Photographs are orientated in the longitudinal plane:
(a view through the diaphysis)



Exposure to emulsion was for 28 days (82 days gestation) or 31 days (140 days gestation)
osteo, osteogenic cell; *ost*, osteoblast; *ostc*, osteocyte; *bm*, bone matrix
Vertical Bar = 100 μm



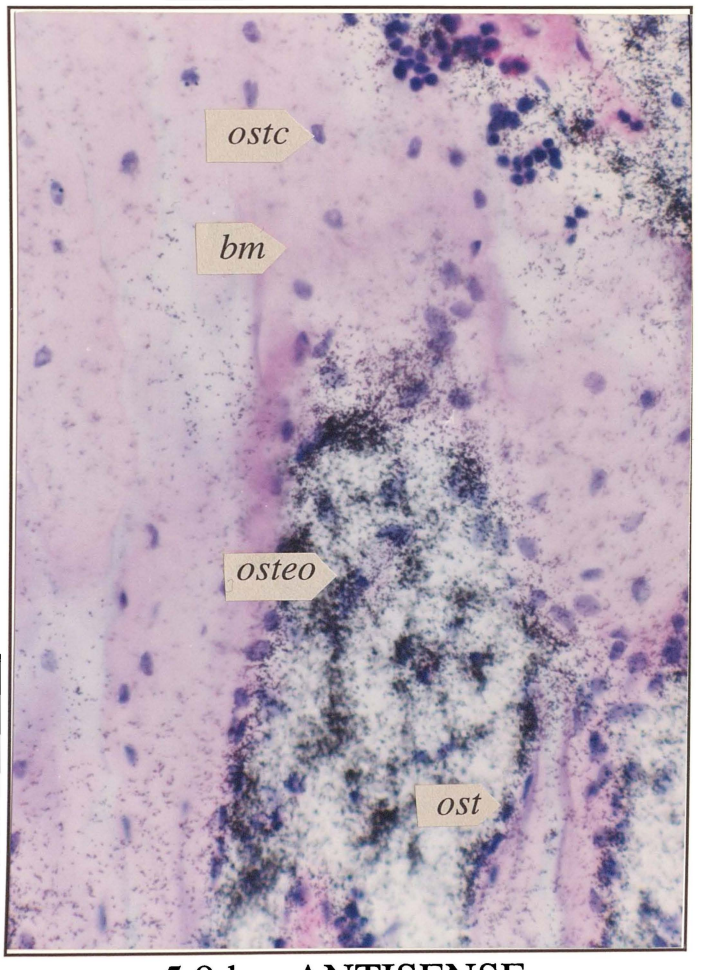
5.9a SENSE



5.9b ANTISENSE



5.9c SENSE



5.9d ANTISENSE

5.3.1.1.3 *Contrast between IGF-I and IGF-II Expression*

Levels of IGF-II mRNA were significantly higher than IGF-I mRNA in all cell types where they were co-localised (Figure 5.10). This difference was found regardless of which bone it was (tibia or femur) and from which age of animal the bone was isolated.

Aside from the abundance of IGF-II mRNA being much greater than IGF-I mRNA, IGF-I and IGF-II mRNA were both found to be widely distributed in bone. They were localised to cells from both cartilage and bone cell lineages in all regions of bone and stages of development. There were similarities and differences in the relative patterns of expression between IGF-I and IGF-II. In general, IGF-II mRNA was predominantly found in less differentiated cells. Particularly in the younger animals, high levels of IGF-II mRNA were found in chondrogenic and osteogenic cells but declined once these cells differentiated. In contrast, relatively high levels of IGF-I mRNA were found in more differentiated cells. Osteoblasts contained the highest level of IGF-I mRNA. While IGF-I and IGF-II mRNA were both high in hypertrophic cells, no IGF-I or IGF-II mRNA could be detected in osteocytes.

Another area of bone where the distribution of IGF-I and IGF-II mRNA differed, was a zone of approximately 50 to 100 cells deep, at the boundary between the growth plate and bone (Figure 5.11). IGF-I expression was highest in this area, whereas at almost all times during foetal development there was an absence of IGF-II mRNA in this area.

5.3.1.2 IGF-I mRNA and IGF-II mRNA in Postnatal Development

Due to the technical difficulties encountered with postnatal sheep bones, analysis of IGF mRNA at the cellular level in these tissues was unable to be accomplished. Discussion of these problems can be found in Chapter 3.

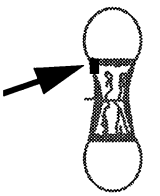
5.3.2 Northern Hybridisation

To complement the *in situ* hybridisation results and determine the size of IGF-I and IGF-II mRNA, Northern hybridisation was performed on RNA extracted from microdissected bones from each age in the foetal and postnatal ontogeny study.

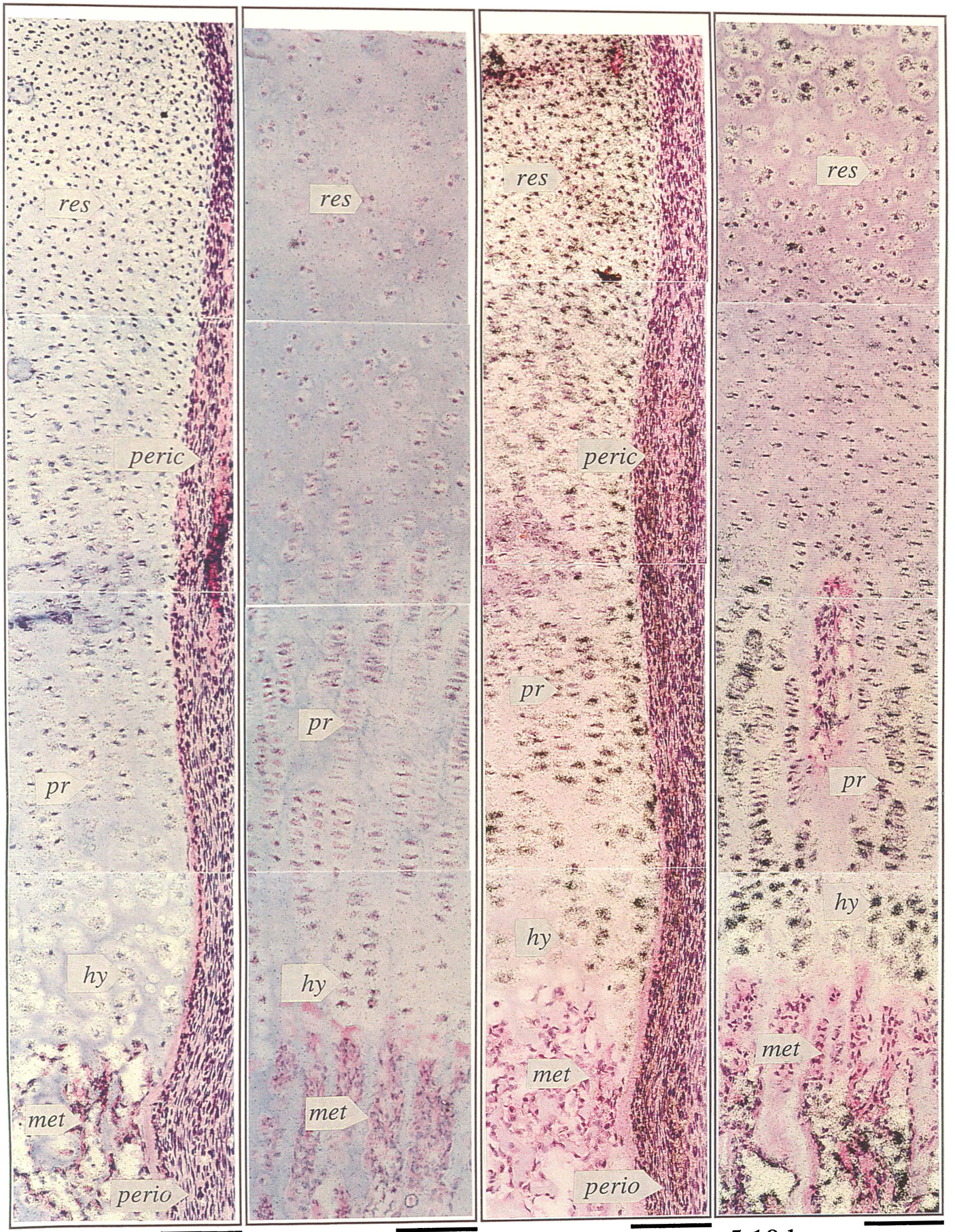
Figure 5.10 (a, b, c, d)

Microautoradiographic images of bone sections from the proximal femur of a 50 day (a and c) and a 140 day (b and d) gestation animal from the foetal ontogeny study. Sections were probed with either an antisense human IGF-I probe (a and b) or an antisense human IGF-II probe (c and d) in adjacent sections.

Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)



Exposure to emulsion was for 69-73 days (IGF-I) or 10-31 days (IGF-II)
perio, periosteum; *peric*, perichondrium; *res*, reserve chondrocyte; *hy*, hypertrophic chondrocyte; *pr*, proliferative chondrocyte; *met*, metaphysis
Horizontal Bar = 100 μm



5.10a
50 days gestation
IGF-I mRNA

5.10b
140 days gestation
IGF-I mRNA

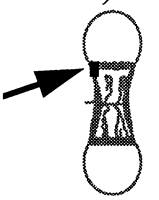
5.10c
50 days gestation
IGF-II mRNA

5.10d
140 days gestation
IGF-II mRNA

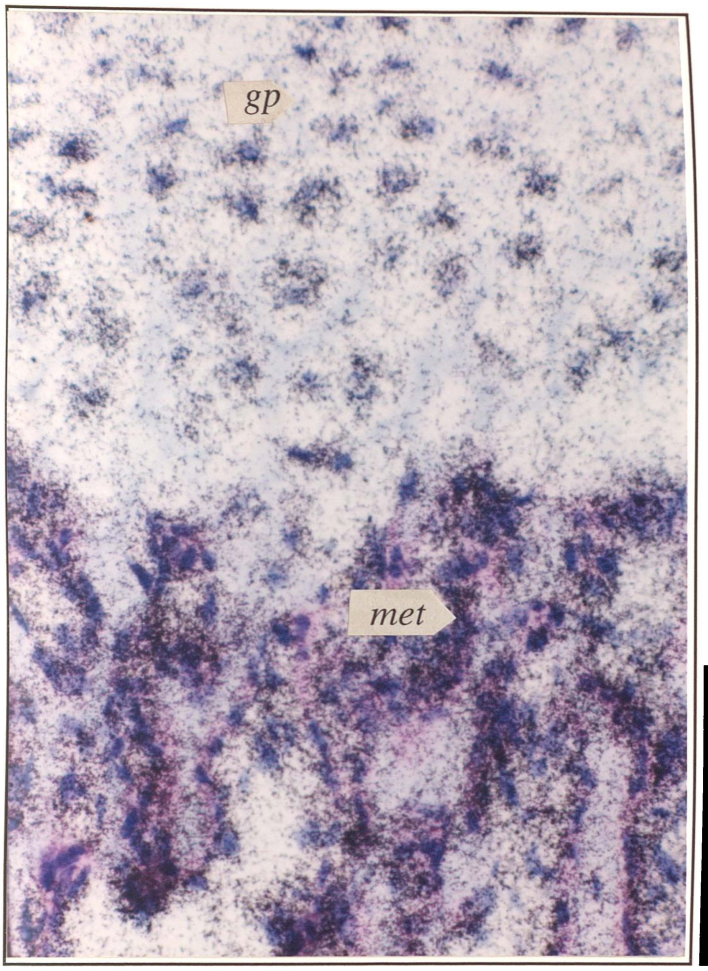
Figure 5.11 (a, b, c, d)

Microautoradiographic images of bone sections from the proximal tibia of an 82 day (a and c) and a 140 day (b and d) gestation animal from the foetal ontogeny study. Sections were probed with either an antisense human IGF-I probe (a and b) or an antisense human IGF-II probe (c and d) in adjacent sections.

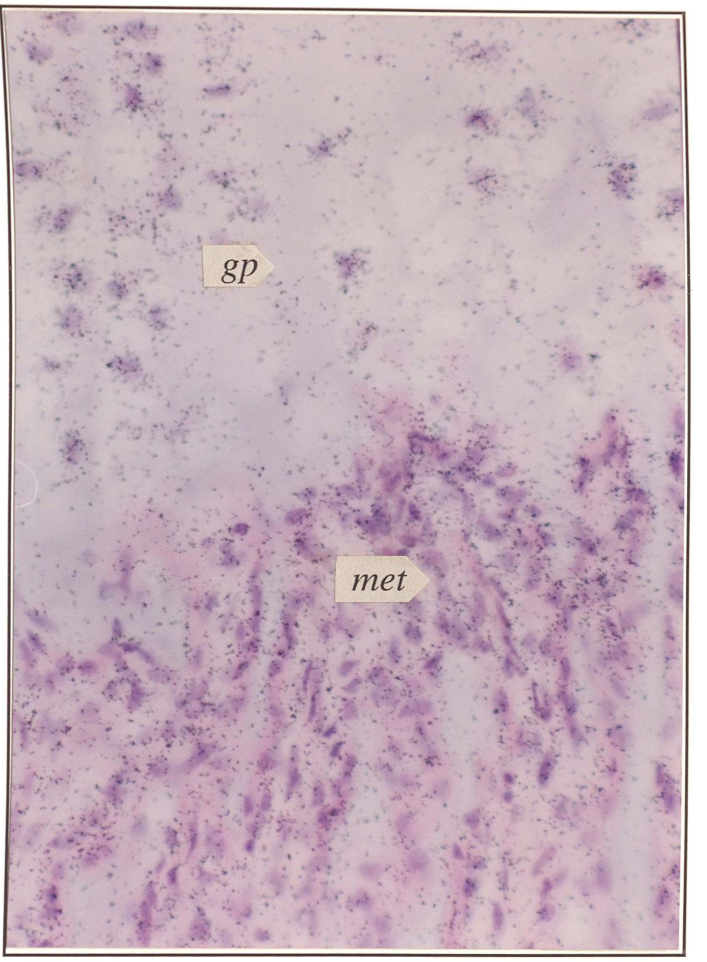
Photographs are orientated in the longitudinal plane:
(a view through the growth plate)



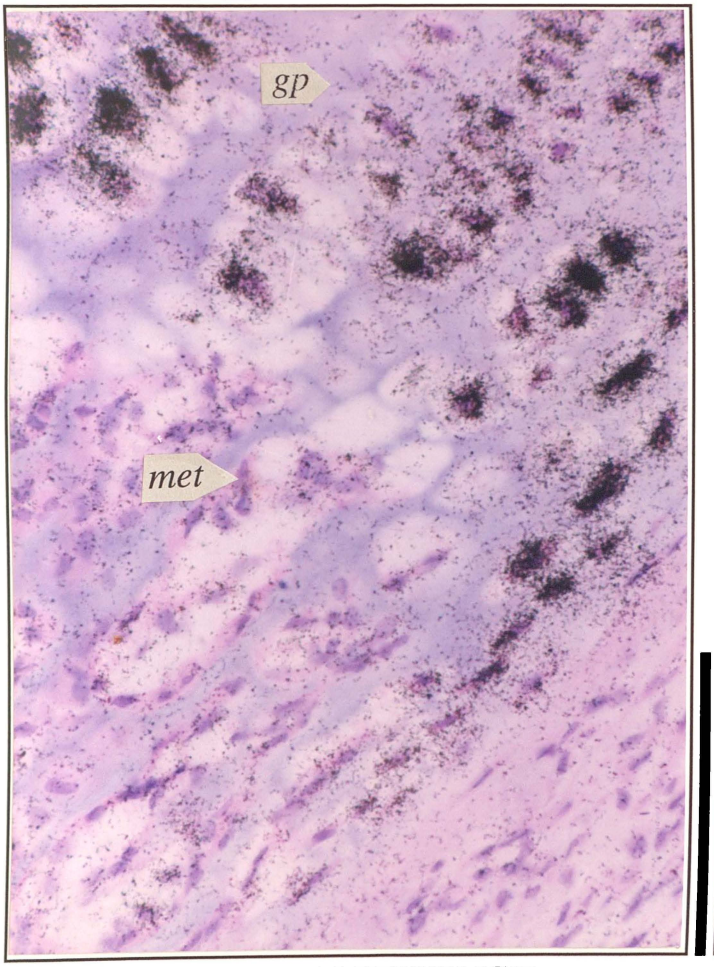
Exposure to emulsion was for 69-73 days (IGF-I) or 24-31 days (IGF-II)
gp, growth plate; *met*, metaphysis
Vertical Bar = 100 μm



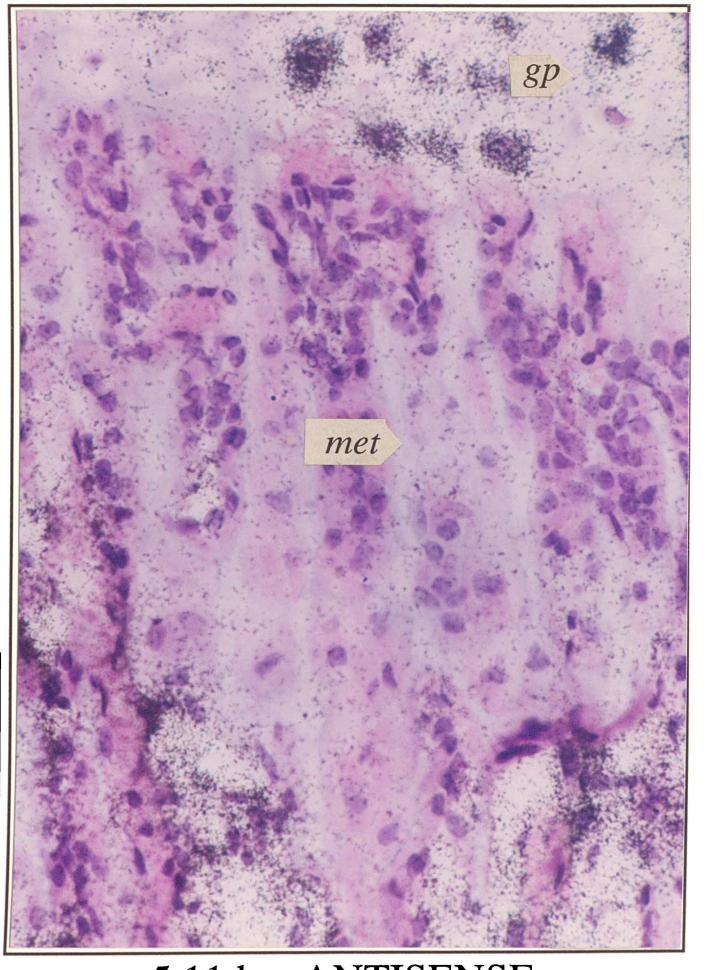
5.11a ANTISENSE



5.11b ANTISENSE



5.11c ANTISENSE



5.11d ANTISENSE

5.3.2.1 IGF-I mRNA in Foetal and Postnatal Development

When Northern hybridisation was carried out using 20 μg of total RNA, IGF-I mRNA could not be detected in any RNA sample isolated from the diaphysis or growth plate region of bone from any age in the foetal or postnatal periods. This result was consistently found regardless of whether the cDNA for probe generation was derived from the human sequence or two non-overlapping ovine sequences.

After long exposures to X-ray film, two faint bands were found in samples at 5.1 and 2.0 kb when using each IGF-I probe (Figure 5.12). These bands were unlikely to be IGF-I mRNA. Based on their size, they were probably the result of cross-hybridisation of the IGF-I probes to 28S and 18S ribosomal RNA. Their location was identical to bands obtained when the blot was subsequently hybridised with a rRNA probe (rRNA bands presented in Figure 5.12).

When the quantity of total RNA analysed in a lane was increased to 45 μg, in addition to the bands believed to be 28S and 18S ribosomal RNA, a faint band at 7.4 kb was found in bone sampled from 50 days gestation and growth plate RNA from 140 days gestation (Figure 5.13). As discussed in Chapter 3, although growth plate RNA was specific to bone, RNA sampled from 50 days gestation was mostly bone with traces of skeletal muscle. Therefore, it cannot be determined whether the IGF-I mRNA detected in this sample was specific to bone. It was possible that most of this IGF-I mRNA was derived from bone since results from *in situ* hybridisation revealed that osteoblasts in bone contain a higher abundance of IGF-I mRNA per cell than skeletal muscle at this age (Table 5.2). However, the amount of mRNA derived from skeletal muscle as a proportion of the total RNA sample could not be determined to confirm this.

The integrity of the IGF-I probes were confirmed by their hybridisation to IGF-I mRNA in RNA isolated from a sheep mammary gland fat pad and adult sheep liver (Figure 5.13). The transcript size of 7.4 kb found for mammary gland fat pad RNA has previously been reported for IGF-I in other tissues such as adult rat liver (Lund *et al.*, 1991; Lund *et al.*, 1986).

Figure 5.12

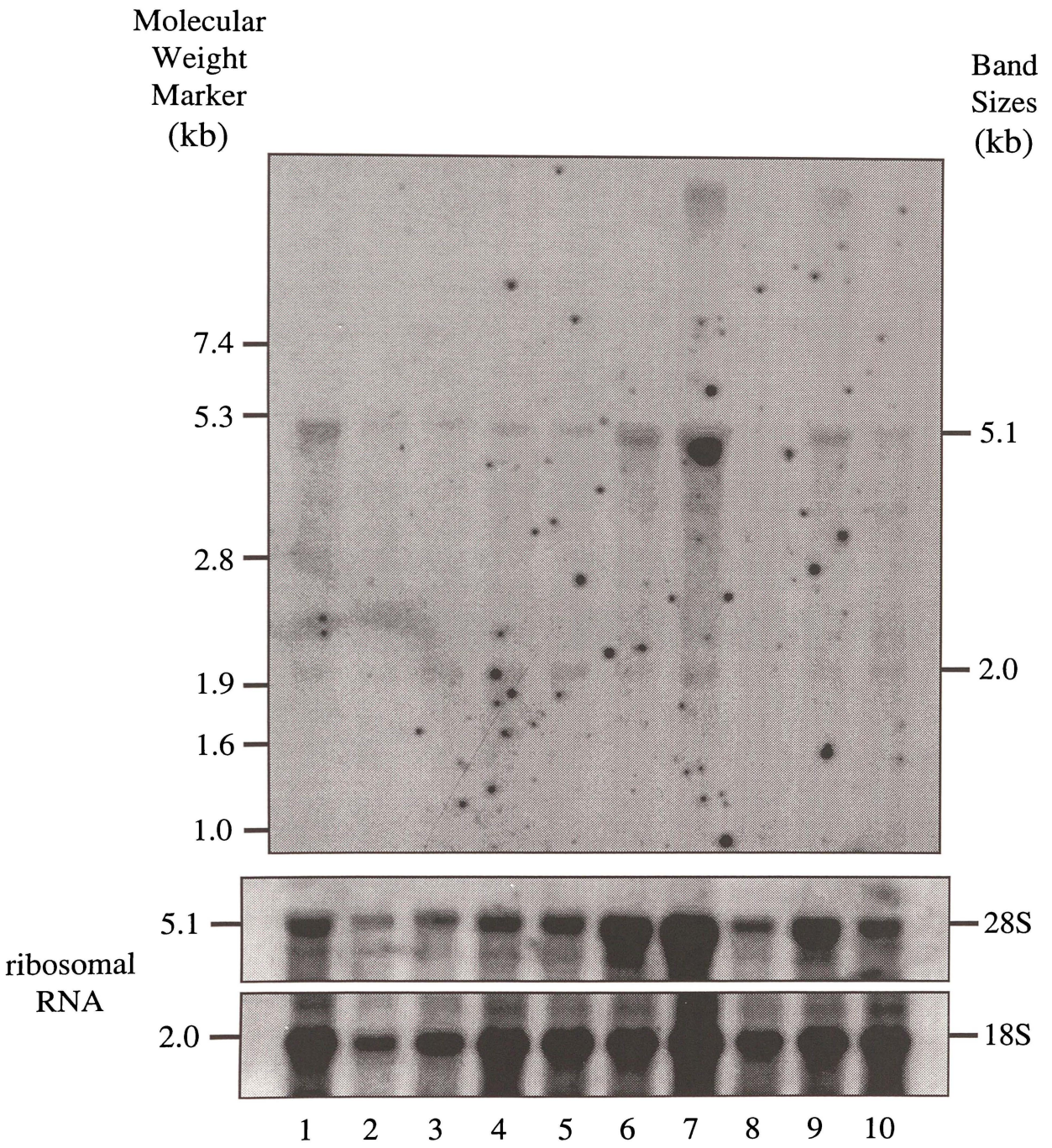
Expression of IGF-I mRNA in bone samples microdissected from whole bone (50 and 62 days gestation) or the diaphysis and growth plate areas of older bones (82, 103, 124 and 140 days gestation).
Total RNA samples (20 μg) were hybridised with a hIGF-I (203 bp) probe

Lower panel shows hybridisation with a ribosomal RNA probe

RNA loaded in each lane was as follows:

1 50 days gestation, fore limb
2 62 days gestation, fore limb
3 82 days gestation, humerus (diaphysis)
4 82 days gestation, humerus (growth plate)
5 103 days gestation, humerus (diaphysis)
6 103 days gestation, humerus (growth plate)
7 124 days gestation, humerus (diaphysis)
8 124 days gestation, humerus (growth plate)
9 140 days gestation, humerus (diaphysis)
10 140 days gestation, humerus (growth plate)

IGF-I blot was exposed for 10 days; rRNA blot was exposed for 3 days



5.12 IGF-I mRNA (hIGF-I probe)

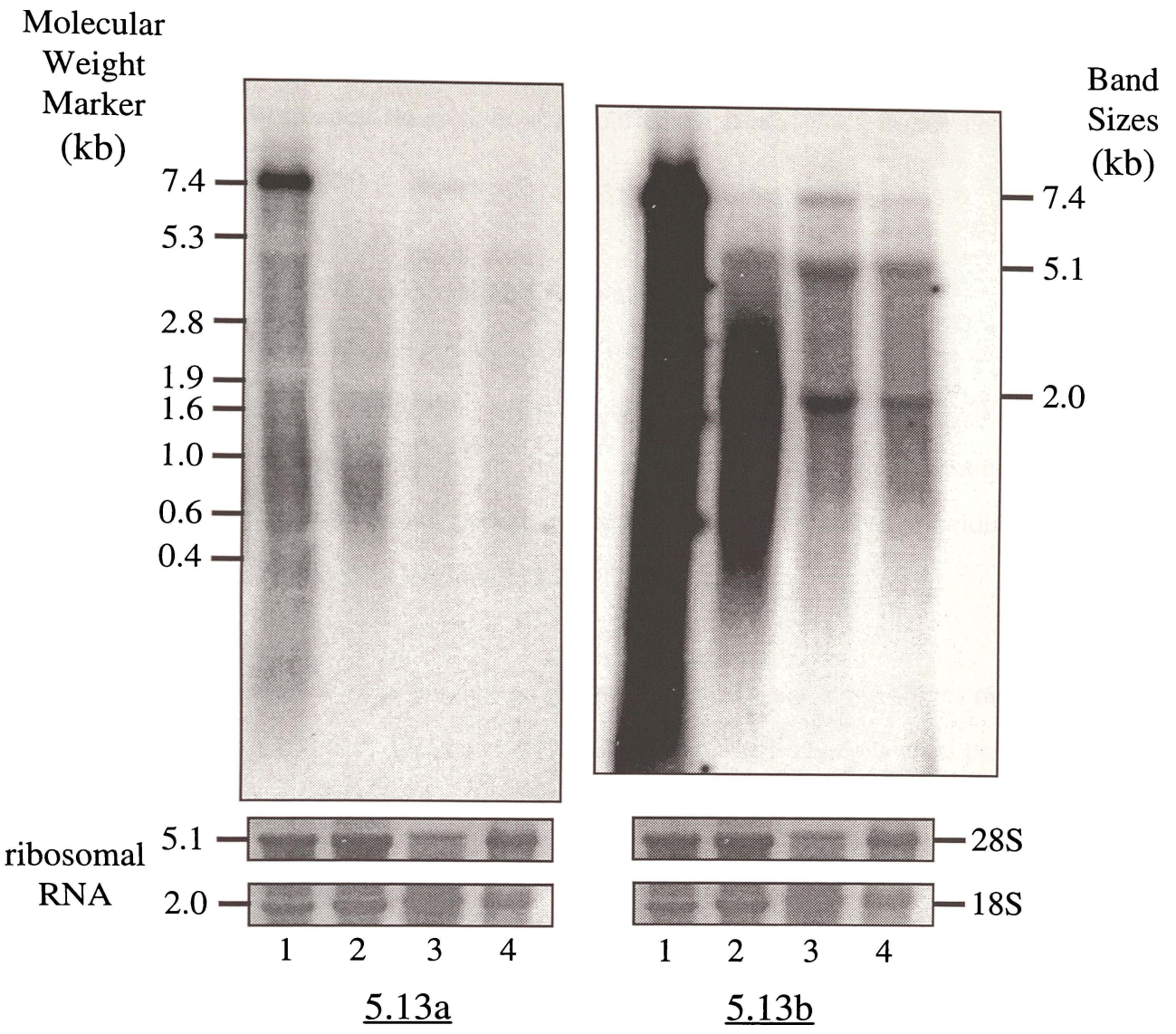


Figure 5.13 (a, b)

Expression of IGF-I mRNA in bone samples microdissected from whole bone (50 days gestation) or the growth plate area of the proximal humerus at 140 days gestation.
Total RNA samples (45 µg) were hybridised with an oIGF-I (264 bp) probe

Lower panel shows methylene blue staining of ribosomal RNA
RNA loaded in each lane was as follows:

1 10 week old sheep mammary gland fat pad
2 adult sheep liver
3 50 days gestation, fore limb
4 140 days gestation, humerus (growth plate)

Blots were exposed for either 1 day (5.13a) or 4 days (5.13b)

Similarly, the pattern of hybridisation observed for adult sheep liver was similar to that found by Wong *et al.* (1989) in adult sheep liver and Godfredson *et al.* (1990) in bovine liver from foetal and adult tissue.

5.3.2.2 IGF-II mRNA in Foetal and Postnatal Development

IGF-II mRNA was abundant in all RNA samples from the foetal period. In postnatal samples where intact RNA could be isolated in sufficient quantities to load on a gel, IGF-II mRNA was also detected. Overall, levels of IGF-II mRNA in the postnatal samples were comparatively lower than the foetal period, when the intensity of IGF-II hybridisation was subjectively compared at equal RNA loadings.

IGF-II mRNA was found to exist as a mixture of transcripts (Figure 5.14). Sizes ranged from 6.4 to 1.0 kb (6.4, 5.2, 4.6, 3.9, 2.0, 1.7 and 1.0 kb). The seven transcripts were present in all RNA samples that were found to contain IGF-II mRNA. The hIGF-II probe and both oIGF-II probes hybridised strongly to these seven transcripts (Figure 5.14). There was however a few minor differences between probes. When samples from the foetal period were hybridised with the hIGF-II probe, not only the seven transcripts listed above were found, but also a weaker band at 0.6 kb (Figure 5.14a). Similarly, the oIGF-II (350 bp) probe hybridised weakly to an additional band at 2.8 kb (Figure 5.14b) and the oIGF-II (388 bp) probe hybridised to additional bands at 2.6 and 1.2 kb (Figure 5.14c).

Regardless of the site (growth plate or diaphysis) or the age of the bone from which RNA was isolated, the same seven transcripts were detected in all samples (Figure 5.14). There was some variation in the relative abundance of each transcript both within a particular RNA sample and between RNA samples (Figure 5.14).

However, these differences were not quantified, as their interpretation and relevance to the level of IGF-II expression in bone was questionable for a number of reasons. A major problem was a lack of a reliable reference on which to base these comparisons. There was experimental variation associated with sampling of the tissue (most RNA samples contained

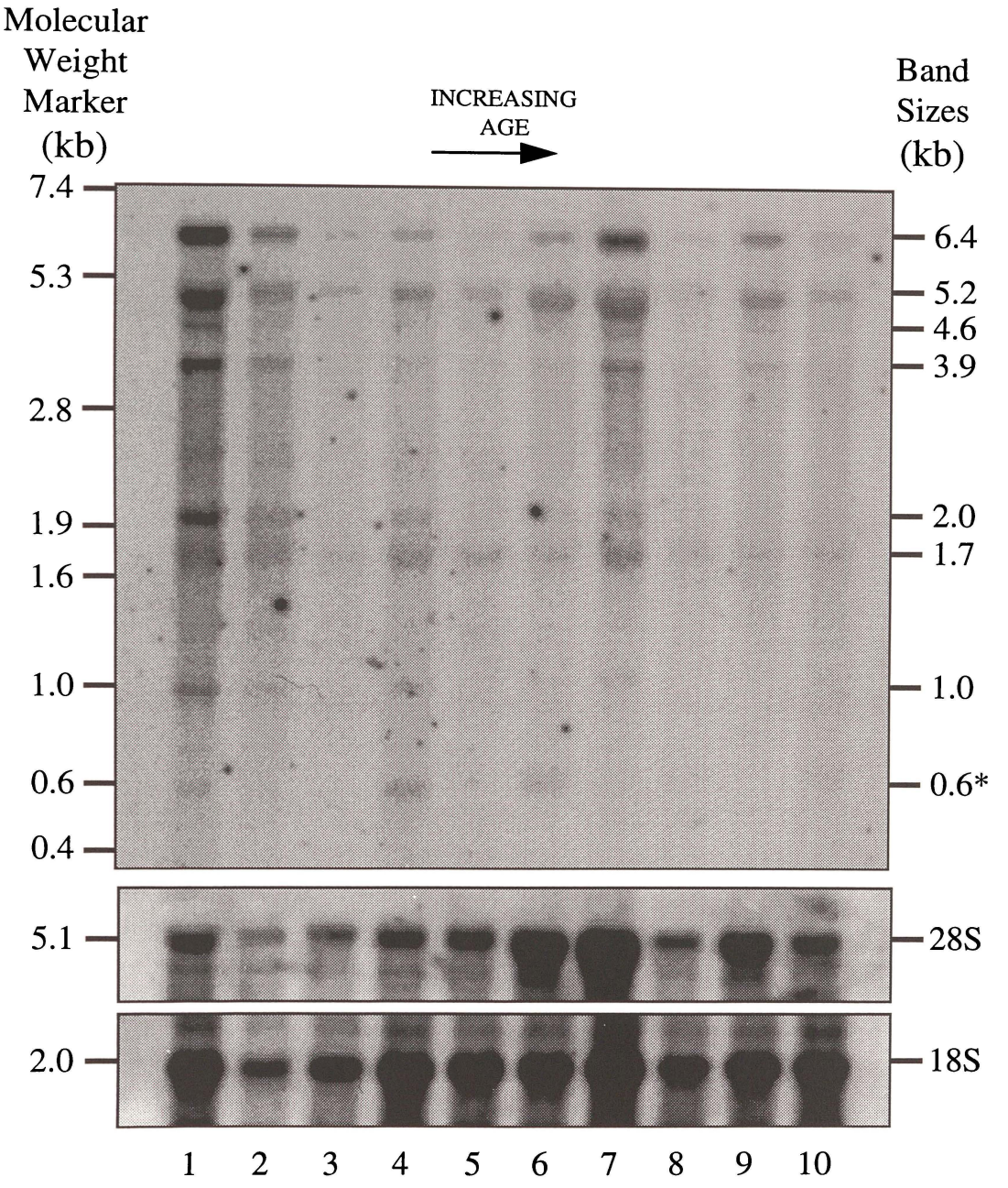
Figure 5.14 (a, b, c)
Expression of IGF-II mRNA in bone samples microdissected from whole bone (50 and 62 days gestation) or the diaphysis and growth plate areas of older bones (82, 103, 124 and 140 days gestation).
Total RNA samples (20 μg) were sequentially hybridised with:
hIGF-II (552 bp) probe (a)
oIGF-II (350 bp) probe (b)
oIGF-II (388 bp) probe (c)

Lower panel shows hybridisation with a ribosomal RNA probe
* indicates a band unique to a particular IGF-II probe

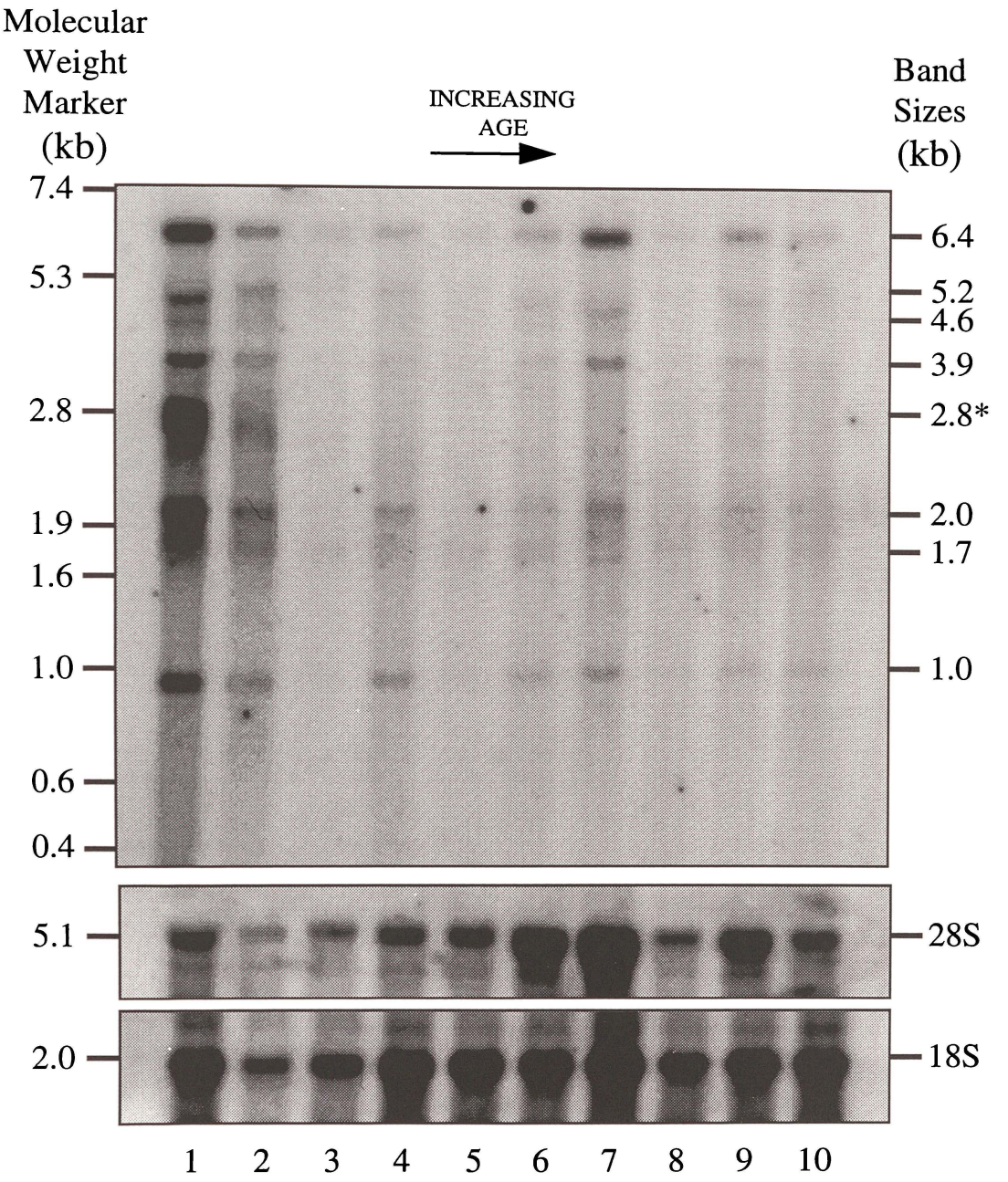
RNA loaded in each lane was as follows:

1 50 days gestation, fore limb
2 62 days gestation, fore limb
3 82 days gestation, humerus (diaphysis)
4 82 days gestation, humerus (growth plate)
5 103 days gestation, humerus (diaphysis)
6 103 days gestation, humerus (growth plate)
7 124 days gestation, humerus (diaphysis)
8 124 days gestation, humerus (growth plate)
9 140 days gestation, humerus (diaphysis)
10 140 days gestation, humerus (growth plate)

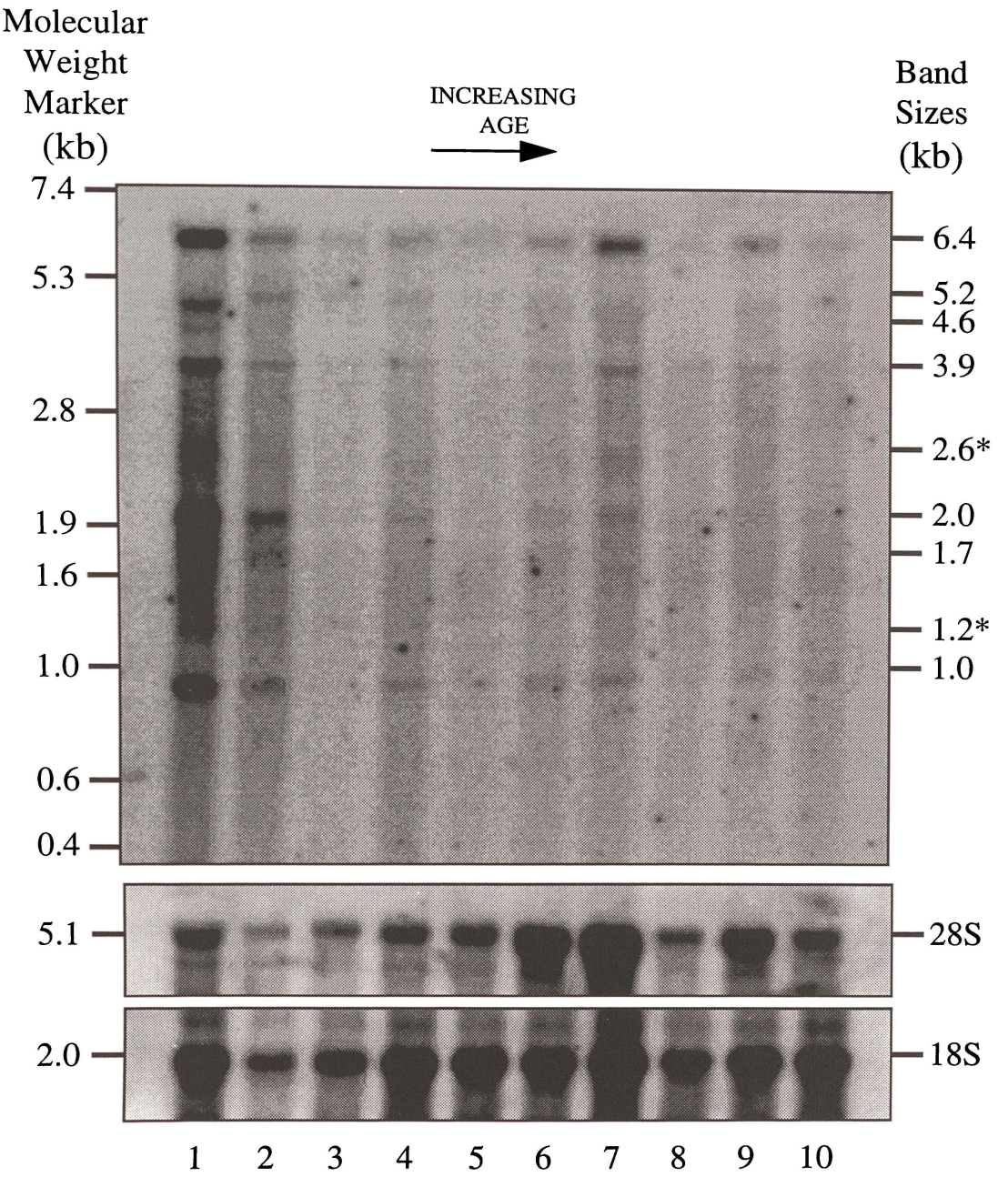
All IGF-II blots were exposed for 7 days; rRNA blot was exposed for 3 days



5.14a IGF-II mRNA (hIGF-II probe)



5.14b IGF-II mRNA (350 bp oIGF-II probe)



5.14c IGF-II mRNA (388 bp oIGF-II probe)

an unknown quantity of skeletal muscle RNA in addition to RNA from bone, Chapter 3), the content of ribosomal RNA and other variations associated with IGF-II mRNA detection.

RNA samples from the earlier foetal ages (50 and 62 days gestation) and the diaphysis of the other foetal animals were found to contain not only RNA from bone, but also RNA from skeletal muscle (Chapter 3). Since IGF-II mRNA was localised to bone and skeletal muscle (as determined by *in situ* hybridisation, Table 5.3), the IGF-II mRNA detected in these RNA samples was likely to be derived from both tissue types. The levels of contaminating skeletal muscle in each RNA sample were unknown. Therefore a comparison in the levels of IGF-II mRNA in bone, both between and within different RNA samples was not able to be determined.

Ribosomal RNA has traditionally been used as an unchanging reference against which mRNA can be compared. However there was clearly variability in rRNA levels between samples in this study. In some samples, the relative levels of 18S and 28S ribosomal RNA were similar, whereas in other samples, there was much less 28S than 18S ribosomal RNA (Figure 5.14). Furthermore, Spanakis and Brouty-Boylé (1994) caution against using only ribosomal RNA as a reference for quantifying expression levels of mRNA, after they performed a detailed analysis of the changes in ribosomal RNA levels under different physiological conditions.

Additional errors that could be associated with quantifying mRNA levels either within or between samples of RNA were the non-linearity of the response of X-ray film to radiation, the limited number of samples that were able to be analysed, variability in the relative hybridisation intensities (assessed by X-ray film) between the three IGF-II probes used to measure IGF-II mRNA and the variability found between repeated hybridisations with the same probe.

Although not quantified for the above reasons, there was an obvious difference in the overall level of IGF-II mRNA between the foetal and postnatal periods (Figure 5.15). IGF-II mRNA tended to be higher in the foetal period than in the postnatal period. However it was only

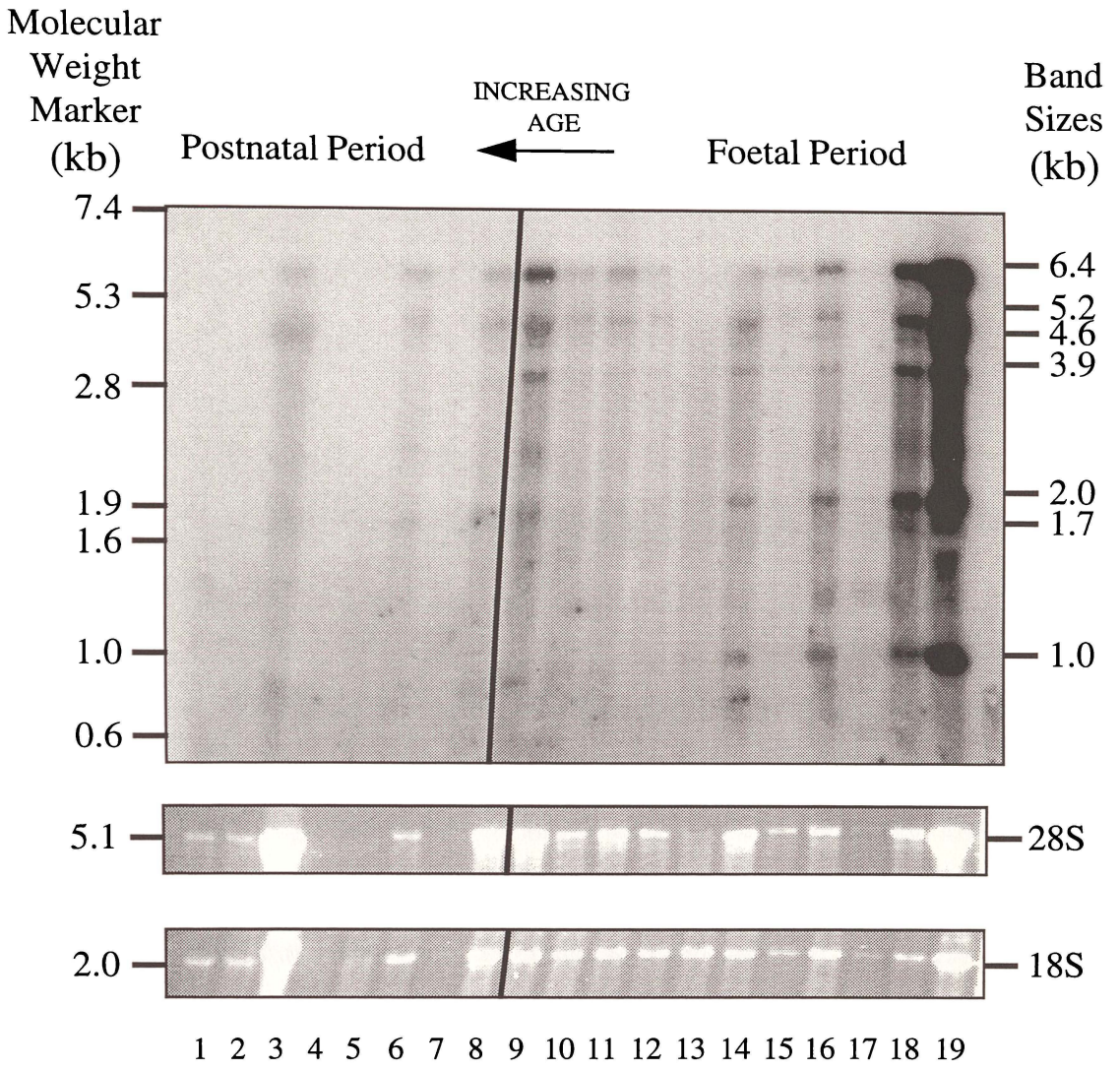


Figure 5.15

Comparison of IGF-II expression between the foetal and postnatal periods.
Expression of IGF-II mRNA in bone samples microdissected from whole bone (50 and 62 days gestation) or the diaphysis and growth plate areas of older bones (82, 103, 124 and 140 days gestation and 2 days, 2 weeks, 6 months and 18 months postnatally).
Total RNA samples (20 µg) were hybridised with a hIGF-II probe.
Lower panel shows ethidium bromide staining of ribosomal RNA

RNA loaded in each lane was as follows:

1	18 month old, humerus (growth plate)
2	18 month old, humerus (diaphysis)
3	6 month old, humerus (growth plate)
4	6 month old, humerus (diaphysis)
5	2 week old, humerus (growth plate)
6	2 week old, humerus (diaphysis)
7	2 day old, humerus (growth plate)
8	2 day old, humerus (diaphysis)
9	140 days gestation, humerus (growth plate)
10	140 days gestation, humerus (diaphysis)
11	124 days gestation, humerus (growth plate)
12	124 days gestation, humerus (diaphysis)
13	103 days gestation, humerus (growth plate)
14	103 days gestation, humerus (diaphysis)
15	82 days gestation, humerus (growth plate)
16	82 days gestation, humerus (diaphysis)
17	62 days gestation, fore limb
18	50 days gestation, fore limb
19	50 days gestation, fore limb (40 µg RNA)

Blot was exposed for 7 days

possible to make this comparison with 5 out of the 8 RNA samples from postnatal bone, since the other 3 samples were either degraded or an insufficient quantity of RNA was available to enable the detection of IGF-II mRNA. This was due to technical difficulties in extracting RNA from the more highly calcified bones found in the postnatal sheep (Chapter 3).

5.4 DISCUSSION

5.4.1 Localisation and Ontogeny of IGF-I and IGF-II mRNA in Growth Plate Cartilage

The source of IGF-I and IGF-II in growth plate cartilage appears to be different. While IGF-II mRNA was found to be highly expressed, IGF-I mRNA was barely detectable throughout foetal development. Although the levels of IGF-I mRNA were extremely low, a single transcript at 7.4 kb was detected by Northern hybridisation and a faint signal was observed by *in situ* hybridisation after long exposures. In contrast, seven different transcripts for IGF-II were found in the growth plate at all ages. These ranged in size from 1.0 kb to 6.4 kb. The overall level of IGF-II mRNA in the growth plate declined at birth to be barely detectable in the growth plate from 6 month old animals.

Despite dramatic differences in their relative abundance, mRNA for IGF-I and IGF-II were similarly distributed throughout the foetal period. Highest levels of IGF mRNA were located in the hypertrophic zone with comparatively less IGF mRNA in chondrocytes from the reserve cartilage and proliferative zones. This pattern of expression matched the pattern of localisation found for IGF-I and IGF-II peptides.

The ability to detect IGF-I mRNA in the growth plate appears to depend on animal age, technique and the interpretation by different authors. While IGF-I expression is reported in some studies, other studies fail to detect IGF-I mRNA at all. Those studies that report the presence of IGF-I mRNA in the growth plate use sensitive techniques such as reverse transcription polymerase chain reaction (RT-PCR), *in situ* hybridisation and solution

hybridisation (Lin and Oberbauer, 1996; Hanna *et al.*, 1995; Lazowski *et al.*, 1994; Nilsson *et al.*, 1990; Isgaard *et al.*, 1988a; Isgaard *et al.*, 1988b). In *in situ* hybridisation long exposures (approximately 40 days or more) are required to produce autoradiographs with a relatively low density of grains (Hanna *et al.*, 1995; Bikle *et al.*, 1994; Lazowski *et al.*, 1994; Nilsson *et al.*, 1990). In adult rat growth plates, low levels of IGF-I mRNA are found to be distributed in most chondrocytes from the proliferative and hypertrophic zones, which is similar to the pattern of expression found in foetal growth plates from the present study (Hanna *et al.*, 1995; Lazowski *et al.*, 1994; Nilsson *et al.*, 1990).

The presence of IGF-I mRNA in the growth plate appears to be developmentally regulated. While IGF-I mRNA is present in growth plates isolated from rats 28 days of age or older (Lin and Oberbauer, 1996; Hanna *et al.*, 1995; Lazowski *et al.*, 1994; Nilsson *et al.*, 1990; Isgaard *et al.*, 1988a; Isgaard *et al.*, 1988b), IGF-I mRNA is below the level of detection in neonatal or foetal growth plates from rats or mice aged between 15 days gestation and 35 days postnatally (Wang *et al.*, 1995; Shinar *et al.*, 1993; Streck *et al.*, 1992; Nilsson *et al.*, 1990; Beck *et al.*, 1988; Beck *et al.*, 1987). An increase in the level of IGF-I mRNA occurs between 28 and 35 days of age in the rat. Nilsson *et al.* (1990) detected a very weak signal for IGF-I mRNA in the growth plates of 10 and 28 day old rats, which became appreciably higher in 35 day old rats. The present study consistently found low levels of IGF-I mRNA in foetal growth plates. However, due to technical difficulties, the predicted postnatal increase in IGF-I mRNA was unable to be determined.

In contrast to this and other studies, Bikle *et al.* (1994) found high levels of IGF-I mRNA in extracts from rat knees (bone and growth plate cartilage) in the late foetal period. The relative level of IGF-I mRNA declined postnatally to reach lowest levels at 6 weeks, after which levels rose again and remained high at all sampling points between 3 and 28 months old. The results from the Bikle study may be different to this and other studies because of the method of analysis used (IGF-I mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which may not be constant) and/or the contribution of IGF-I mRNA from bone (which was not present in the other studies) (Bikle *et al.*, 1994).

A number of *in vitro* studies have confirmed that local production of IGF-I by chondrocytes from the foetal or neonatal periods is extremely low. Chondrocytes derived from postnatal rabbit articular cartilage or embryonic chick cartilage have been shown to produce IGF-I in serum free media (Froger-Gaillard *et al.*, 1989; Burch *et al.*, 1986). However, after three days in culture these chondrocytes produce only 259 pg/ml media (embryonic chick cartilage) or a cumulative production of 0.63 ng (postnatal rabbit articular chondrocytes) (Froger-Gaillard *et al.*, 1989; Burch *et al.*, 1986). Although Burch *et al.* (1986) found IGF-I in the media, there was no IGF-I in the cartilage itself. IGF-I is too low to be detected in the media of chondrocytes derived from foetal sheep growth plates or mouse embryonic limbs grown under serum free conditions (Bhaumick, 1993; Hill and De Sousa, 1990a). This is in agreement with results found in the present study.

The presence of IGF-I in serum free media, however, does not necessarily indicate local production of IGF-I. What appeared to be IGF-I synthesis in cultured endothelial cells (presence of IGF-I mRNA, presence of IGF-I in serum free media) in a recent study turned out to be sequestration of IGF-I into these cells prior to serum free conditions and release of intact peptide into the media under subsequent serum free conditions (Gajdusek *et al.*, 1993). Therefore, those studies that have detected low levels of IGF-I in cultured chondrocytes may have measured IGF-I uptake from the serum they were cultured in and not local production of IGF-I. Further investigation is needed to determine if the low level of IGF-I produced *in vitro* represents synthesis of IGF-I by chondrocytes.

It is unclear whether the very low level of IGF-I expression found in the growth plate in the present study is either sufficient to account for distribution found for IGF-I peptide, or sufficient to produce the level of IGF-I required to generate a biological response. In tissues such as liver it is believed that a low steady state level of IGF-I mRNA correlates with a low level of IGF-I production, with factors such as protein restriction affecting the rate of transcription and not translation (Thissen and Underwood, 1992a). However, Gajdusek *et al.* (1993) found IGF-I mRNA in endothelial cell culture in the absence of endogenously

produced IGF-I. Similarly, Haselbacher *et al.* (1987) found no correlation between IGF-II mRNA transcript levels and IGF content in tumors.

Overall, very little is known about the biosynthesis of IGF-I or its processing into mature peptide *in vitro* or *in vivo*. It is also unclear which of the various IGF-I transcripts are translated into protein. In the present study the size of IGF-I mRNA in foetal sheep growth plates was found to be 7.4 kb. This corresponds to the predominant IGF-I transcript found in a range of rat tissues including different regions of bone such as the diaphysis and metaphysis microdissected from the neonate (Shinar *et al.*, 1993; Lund *et al.*, 1991). Only approximately 2 kb of the 7.4 kb is translated into IGF-I peptide (Lund *et al.*, 1991). The rest is believed to be largely 3′ untranslated sequence. This large 3′ untranslated sequence is likely to influence mRNA stability which is an important determinant of the rate of protein synthesis. In those tissues that produce a range of transcript sizes for IGF-I, Lund *et al.* (1991) found that the larger 7.0 - 7.5 kb transcripts had a longer half life both *in vitro* and *in vivo* compared to the smaller transcripts. A single larger sized IGF-I transcript in the foetal growth plate may allow a fewer number of mRNAs to support the synthesis of a greater number of IGF-I peptide molecules because of the longer half life.

In addition to heterogeneity at the 3′ end of IGF-I mRNA, there is also 5′ heterogeneity resulting from different transcription start sites on leader exon 1 or leader exon 2 and alternative splicing of exon 5. Although it is unclear whether the 7.4 kb transcript identified in the present study corresponds to class 1 Ea transcripts, class 1 Eb transcripts, class 2 Ea transcripts or class 2 Eb transcripts, a recent study by Lin and Oberbauer (1996) found that the IGF-I class 1 Ea mRNA are the most abundant transcripts expressed in the growth plate of 28 day old rats. Similarly, Bikle *et al.* (1994) found predominantly IGF-I Ea mRNA in RNA from rat bone analysed for class Ea or Eb transcripts using specific probes in a solution hybridisation/RNase protection assay. This particular class of IGF-I transcripts appears to be low in the reserve and hypertrophic zones of the growth plate but twenty fold higher than the other class of transcripts in the proliferative zone (Lin and Oberbauer, 1996).

IGF-I class 1 Ea transcripts contain exons 1, 3, 4 and 6 of the IGF-I gene. It has been postulated that the exclusion of exon 5 is important for targeting this class of transcripts to an autocrine or paracrine pathway of action in tissues where it is produced (Lund *et al.*, 1991; Hoyt *et al.*, 1988). However, further investigation is required to determine the class of IGF-I transcripts produced in the foetal sheep growth plate. Unlike other species, the sheep IGF-I gene has an additional exon (designated exon W) upstream of exon 1 that is spliced to exon 3 (Ohlsen *et al.*, 1993). Usage of this exon may produce an additional IGF-I mRNA species with properties unique to sheep IGF-I action. A knowledge of which class of IGF-I transcripts exist in the ovine foetal growth plate may not only indicate the likely target of IGF-I actions but will also assist in the identification of factors that could potentially influence transcription of IGF-I (Lund *et al.*, 1991).

Levels of IGF-I peptide were not measured in growth plates from the present study. However, IGF-I is nine times lower than IGF-II in human adult bone (Frolik *et al.*, 1988; Mohan *et al.*, 1988a) and between 1.6 and 7.8 times lower than IGF-II in a range of other human foetal tissues (Hill and De Sousa 1990a). Since the vast majority of IGF-I present in cartilage is believed to be due to IGF-I storage in the extracellular matrix (Rosen *et al.*, 1994; Hauschka *et al.*, 1986) for which IGF BPs and extracellular matrix proteins have been postulated to play a role (Jones and Clemmons, 1995; Hodgkinson *et al.*, 1995a; Hodgkinson *et al.*, 1995b; Jones *et al.*, 1993), any measure of IGF-I content in cartilage at a given time is likely to reflect accumulated IGF-I, with only a minor contribution from steady state IGF-I production. It therefore seems possible that the low levels of IGF-I mRNA found in the growth plate could be sufficient to account for the IGF-I found in growth plate cartilage. Furthermore, IGF-I mRNA and IGF-I peptide were found to have a similar pattern of localisation in the present study. These results support a potential paracrine/autocrine role for IGF-I in foetal sheep growth plates.

However, the presence of IGF-I in the growth plate could also be due to production and transport from another location. There is evidence to support a role in the postnatal rat growth plate for IGF-I derived from the circulation (Hunziker *et al.*, 1994; Guler *et al.*, 1988;

Scheiwiller *et al.*, 1986; Schoenle *et al.*, 1982). It is therefore possible that circulating IGF-I also has a role in the foetal sheep growth plate. Not only is IGF-I found in sheep plasma throughout the foetal period (Mesiano *et al.*, 1989; Gluckman and Butler, 1985; Van Vliet *et al.*, 1983), but levels correlate positively with foetal weight (Carr *et al.*, 1995). Part of the increased foetal weight could be due to the actions of circulating IGF-I on the growth plate and consequently on bone growth in foetal sheep. IGF-I could also be transported from other locations within bone. As discussed below, IGF-I mRNA was abundant in cells located in the metaphysis (which is adjacent to the growth plate) and in osteoblasts throughout bone. Also, IGF-I mRNA along with IGF-I peptide was localised to putative chondrogenic cells residing in the perichondrium. Other studies have also demonstrated the presence of IGF-I mRNA in these locations in rat and mouse growth plates from the foetal and neonatal periods (Wang *et al.*, 1995; Shinar *et al.*, 1993).

In contrast to IGF-I, IGF-II mRNA was abundant in sheep growth plates throughout the foetal period in a similar pattern to that found for embryonic, foetal and neonatal rat and mouse growth plates (Wang *et al.*, 1995; Shinar *et al.*, 1993). In keeping with the proposal that IGF-II has a major paracrine or autocrine mode of action in the foetus (Cohick and Clemmons, 1993), IGF-II mRNA and IGF-II peptide were found co-localised in the same regions of the growth plate throughout foetal development. Evidence from other studies suggest that the presence of IGF-II mRNA in chondrocytes correlates with production of IGF-II. Lee *et al.* (1990) showed that IGF-II mRNA and the pre-pro form of IGF-II were co-localised in growth plate chondrocytes, indicating that translation of IGF-II was occurring in these cells. Likewise chondrocytes grown in cell culture have been shown to release IGF-II into the media under serum free conditions (Bhaumick, 1993; Hill and De Sousa, 1990a; Froger-Gaillard *et al.*, 1989). It is therefore probable that locally produced IGF-II accounts for most of the IGF-II present in cartilage.

Based on *in vivo* evidence, it is unlikely that circulating IGF-II makes a major contribution to the biological actions of IGF-II reported in the growth plate. Compared to IGF-I, circulating IGF-II has only minimal effects on longitudinal bone growth. In mice containing an IGF-II

transgene, no effect on overall body weight was found despite 2-3 fold higher serum levels of IGF-II (van Buul-Offers *et al.*, 1995; Wolf *et al.*, 1994; Ward *et al.*, 1994). Although there was variable disproportionate growth between some organs postnatally in the IGF-II transgenic animals, bone growth parameters were not significantly different between transgenic mice and controls (Wolf *et al.*, 1994). When compared equivalently, IGF-II is either much less potent than IGF-I or has no effect at stimulating an increase in growth plate width in hypophysectomised rats infused with IGF-I or IGF-II (Glassock *et al.*, 1992; Schoenle *et al.*, 1985). Furthermore, despite the high levels of IGF-II present in foetal plasma, circulating levels of IGF-I but not IGF-II correlate with foetal weight in the ovine foetus suggesting that circulating IGF-II has no effect in bone (Carr *et al.*, 1995).

In the present study, multiple transcripts for IGF-II were detected in growth plates microdissected from sheep bone. The same seven transcripts sized between 1.0 and 6.4 kb were present from 50 days gestation through to birth and postnatally in those growth plate samples where IGF-II could be detected. Results from the two youngest ages represented IGF-II mRNA in growth plate, bone and skeletal muscle since the growth plate was too small to be separated at these time points. While the same number of transcripts roughly of a similar size have been described in a range of other tissues from the ruminant (Listrat *et al.*, 1994; Boulle *et al.*, 1993; Delhanty and Han, 1993; O'Mahoney *et al.*, 1991), fewer IGF-II transcripts are found in foetal rat tissues including bone (Bikle *et al.*, 1994; Shinar *et al.*, 1993; Han *et al.*, 1992; Soares *et al.*, 1986). This is likely to be due to differences in the organisation of the sheep and rat IGF-II genes. While the sheep IGF-II gene consists of 10 exons and 4 promoters (P1-P4), the rat IGF-II gene has only 6 exons and 3 promoters (Ohlsen *et al.*, 1994).

Heterogeneity in the IGF-II mRNA population originates from the use of multiple promoters, alternate splicing, and differential use of polyadenylation sites (Nielsen *et al.*, 1995; Ohlsen *et al.*, 1994). Particular promoters in the IGF-II gene have been shown to be developmentally regulated. It is believed that promoters 3 and 4 are the major promoters used in the foetus, whereas promoters 1 and 2 appear to be largely utilised postnatally (Ohlsen *et al.*, 1994; Ikejiri

et al., 1991; Holthuizen *et al.*, 1990). In the rat, usage of the P2 promoter and exon 4 corresponds to a 5 kb fragment on a Northern blot (Holthuizen *et al*, 1990). In sheep, usage of P1 results in a 5.3 kb transcript in postnatal sheep liver, whereas P3 and P4 usage produces transcripts of 6.0 kb and 4.8 kb, respectively (Demmer *et al.*, 1993). In contrast, RNA from bovine foetal liver and skeletal muscle express transcript sizes of 5.2, 3.4, 2.8 and 2.1 kb when expression is driven by P3, whereas transcription from P4 produces 4.0 and 1.0 kb fragments in the same tissues (Boulle *et al.*, 1993). Clearly there are differences in the reported sizes for transcripts produced from particular promoters between these two studies, which is unlikely to be due to differences between ovine and bovine species because of the similarity in their gene structure. The difference in sizes could reflect the usage of alternative polyadenylation sites.

While four of the IGF-II mRNA species found in the present study are likely to be the result of usage of P3 and P4 based on their size, the other three transcripts have yet to be characterised. It will be essential to identify which promoters are utilised for particular IGF-II transcripts to enable identification of factors that control IGF-II expression and to determine the significance of the particular transcripts produced in the growth plate.

The presence of a high level of IGF-II mRNA and very low IGF-I mRNA in foetal growth plates in the present study is similar to that which has been found in various other foetal tissues (Singh *et al.*, 1991; Norstedt *et al.*, 1988). Often this pattern of expression is reversed in the postnatal period. In many tissues a foetal-to-adult switch in IGF mRNA production occurs where IGF-II mRNA declines and IGF-I mRNA levels rise (Norstedt *et al.*, 1988). In sheep this fall occurs at birth in many tissues (Delhanty and Han, 1993; Hodges, 1992; Han *et al.*, 1992; O'Mahoney *et al.*, 1991) whereas in the rat it occurs in the neonatal period in most tissues at approximately 20 days of age (Beck *et al.*, 1988, Norstedt *et al.*, 1988; Singh *et al.*, 1991; Brown *et al.*, 1986). A similar fall in the level of IGF-II mRNA postnatally was observed in the present study in RNA extracted from the growth plate. However, due to technical difficulties, foetal IGF-II mRNA levels were only able to be compared to levels at 6 months postnatally.

In sheep, Broad and Davies (1981) found that bone grew faster prenatally than postnatally relative to carcass weight. Reduced IGF-II expression may reflect reduced activity in the growth plate between the prenatal and postnatal periods.

The higher abundance of IGF-II mRNA in the foetal period reflects the tendency for IGF-II to dominate IGF activity in the growth plate prenatally compared to postnatally. IGF-II is much more successful than IGF-I at stimulating clonal growth of chondrocytes isolated from the foetal period compared to the postnatal period (Vetter *et al.*, 1986). However, at the cellular level both IGF-I and IGF-II peptides were co-localised with low levels of IGF-I mRNA and high levels of IGF-II mRNA. The relationship between IGF-I and IGF-II in the foetal growth plate given that the biological actions of both IGF-I and IGF-II occur largely through the Type I IGF receptor requires further investigation (Schlessinger *et al.*, 1995; Jones and Clemmons, 1995; Lau *et al.*, 1994; Liu *et al.*, 1993).

While these results suggest that a paracrine or autocrine mode of action for IGF-II dominates in the foetal period, it is less clear whether locally produced IGF-I is sufficient to account for all the IGF-I peptide found in the foetal growth plate. The contribution of circulating IGF-I to the pool of IGF-I peptide found in the growth plate will be discussed in the next Chapter.

5.4.2 Localisation and Ontogeny of IGF-I and IGF-II mRNA in Bone

IGF-I mRNA was more abundant in bone compared to growth plate cartilage, while the relative levels of IGF-II mRNA were similar to that which was found in chondrocytes from the growth plate. IGF-I and IGF-II were differentially expressed in cells of the osteoblast lineage. IGF-I was localised to more mature osteoblasts in the bone cell lineage, whereas IGF-II mRNA was found in less mature osteoblasts.

This differential pattern of expression was most pronounced in the metaphysis adjacent to the growth plate. IGF-I mRNA but not IGF-II mRNA was located in this area throughout foetal development. While the identity of individual cells was not able to be determined with

certainty, this area is characterised by cartilage degradation and active bone formation which involves osteoblasts and angiogenesis. It is tempting to speculate that important effects of IGF-I on bone growth could be carried out via IGF-I action in this zone. Given the importance ascribed to the process of vascularisation by Caplan and Pechak (1987) in longitudinal bone growth, and the known actions of IGF-I on angiogenesis, cartilage degradation and bone formation, the region adjacent to the metaphysis needs to be investigated more rigorously.

While the presence of IGF-I mRNA and IGF-II mRNA has previously been localised to osteoblasts from foetal and postnatal bone (Wang *et al.*, 1995; Shinar *et al.*, 1993) few studies have defined in which stages of osteoblast differentiation IGF-I and IGF-II are expressed. Only one study has characterised the pattern of IGF-I expression during osteoblast proliferation and differentiation. In contrast to the present study, which showed the presence of IGF-I mRNA in more mature osteoblasts, Birnbaum and Bowsher (1993) found IGF-I mRNA in less mature osteoblasts, with levels declining once proliferation had ceased. The specific stages of osteoblast differentiation were unable to be fully characterised in the present study. Further investigation using appropriate markers for osteoblast development will need to be undertaken to understand the significance of the differential pattern of expression for IGF-I and IGF-II observed in the present study (Owen *et al.*, 1990; Aronow *et al.*, 1990; Caplan, 1990).

IGF-I mRNA was found to exist as a 7.4 kb fragment in agreement with that which has previously been reported for different regions of neonatal rat bone (Shinar *et al.*, 1993). In contrast, multiple species of IGF-II mRNA ranging in size from 1.0 to 6.4 kb were found in the diaphysis. While various regions of rat bone have also been shown to contain a number of different IGF-II transcripts (Shinar *et al.*, 1993; Bikle *et al.*, 1994) different sizes and a fewer number of IGF-II mRNA species were found in these studies. This is likely to be due to the differences in the rat and sheep IGF-II genes, which was discussed in the previous section. It must be remembered that the RNA samples from the diaphysis are not specific to bone. They all contained unknown levels of RNA from skeletal muscle which has previously been shown

to contain IGF-I and IGF-II mRNA (Hodges, 1992). However, the transcripts detected for IGF-I and IGF-II in RNA extracted from the diaphysis of foetal bone were the same ones found in RNA from the growth plate. Further investigation is required to fully characterise the significance of each of these transcripts.

A greater abundance of IGF-I mRNA was found in bone cells compared to cartilage cells in this and other studies (Wang *et al.*, 1995; Shinar *et al.*, 1993). This suggests there is greater potential for paracrine or autocrine IGF-I actions in bone compared to cartilage. In support of this, most studies find production of IGF-I in cultured osteoblasts derived from both the foetal and postnatal periods under serum free conditions (Scharla *et al.*, 1991; Mohan *et al.*, 1990; Ernst and Froesch, 1988; Mohan *et al.*, 1988b; Canalis *et al.*, 1988). Although this does not rule out the possibility that circulating IGF-I has actions in foetal bone metabolism, an autocrine or paracrine mode of action in foetal bone appears to be more certain in bone than in the growth plate.

CHAPTER 6

DISTRIBUTION OF BLOOD-DERIVED IGF-I IN FOETAL BONE

6.1 INTRODUCTION

It is clear from *in vivo* and *in vitro* studies that IGF-I is an important factor involved in foetal and postnatal skeletal growth. Both locally produced and circulating IGF-I are potential contributors to the effects observed when there is an absence of IGF-I (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). However, the relative importance of each source of IGF-I to skeletal growth is controversial. Models to explain the postnatal growth promoting actions of IGF-I in bone have either emphasised the importance of endocrine derived IGF-I or paracrine/autocrine derived IGF-I.

The original somatomedin hypothesis proposed that IGF-I was released from the liver in response to GH stimulation and was transported via the circulation to distant sites of action in an endocrine fashion. This was based on the observation that GH stimulated sulphate uptake in cartilage *in vivo* but not *in vitro*, implying that GH effects were mediated by another serum factor (Salmon and Daughaday, 1957). This factor was later identified as IGF-I. Although recent evidence has shown that this hypothesis does not explain all GH or IGF-I actions in bone, support for an endocrine role for IGF-I has consistently been found.

Postnatally, an increase in circulating IGF-I levels in GH-deficient and intact animals has been shown to stimulate skeletal growth parameters (Martínez *et al.*, 1994; Guler *et al.*, 1988; Scheiwiller *et al.*, 1986; Schoenle *et al.*, 1982). Similarly, infusing IGF-I into the arterial supply of one hind limb results in growth effects on cartilage and bone in the targeted limb compared to the contralateral limb (Spencer *et al.*, 1991; Schlechter *et al.*, 1986).

The specific target sites for circulating IGF-I in bone are unknown. Furthermore, the importance of circulating IGF-I in the foetal period has not been investigated. Results from the previous two chapters show that IGF-I is widely distributed in bone throughout the sheep foetal period. This is consistent with the range of effects reported for IGF-I in areas of cartilage and bone. However, IGF-I mRNA, although widely distributed, was barely detectable. It is unknown whether the particularly low level of IGF-I expression found in the growth plate is sufficient to account for the presence of IGF-I and consequently the growth promoting effects of IGF-I.

It is possible that IGF-I is also derived from the circulation and is delivered to specific sites within bone to fulfill an endocrine mode of action. This mode of action may be particularly important in foetal bone, where unlike postnatal bone, significant levels of IGF-I mRNA have not been found in this or any other study (Wang *et al.*, 1995; Shinar *et al.*, 1993; Nilsson *et al.*, 1990; Beck *et al.*, 1988; Beck *et al.*, 1987; Han *et al.*, 1987).

To test whether circulating IGF-I is capable of entering foetal bone, and to determine which areas in bone are potential targets for endocrine derived IGF-I, the transport of ^{125}I-IGF-I from the circulation into bone was investigated in foetal sheep.

6.2 METHODS

Six foetuses between 133 and 140 days gestation were administered a 1 ml bolus of ^{125}I-IGF-I (~55 μCi; ~430 ng IGF-I) into the carotid artery, as described in Chapter 2. This represented less than 1% of the circulating pool of IGF-I. Surgery was performed over three days with

two animals undergoing treatment each day. All animals received tracer from the same batch of ^{125}I-IGF-I, iodinated two days prior to the first day of surgery. Radioactive measurements were equalised for radioactive decay by correcting all results relative to the first day of surgery.

Three animals (one per day) were sacrificed 12 minutes following injection of ^{125}I-IGF-I. These will be referred to as the 12 minute animals. Prior to sacrifice, foetal blood was sampled at 1 and 10 minutes. Additionally, maternal blood was sampled at 10 minutes, to verify that no infused radioligand reached the maternal circulation. Due to an error in measuring the correct dose of ^{125}I-IGF-I, very low levels of ^{125}I-IGF-I were delivered into the circulation of one of the 12 minute animals. Although excluded from analyses of radioactive content, tissue sections from this animal served as a negative control for radioactive detection, as appropriate.

The other three animals (one per day) were sacrificed 180 minutes following ^{125}I-IGF-I infusion. These will be referred to as the 180 minute animals. Blood was sampled at 1, 2, 5, 10, 20, 60 and 180 minutes. To verify that no infused radioligand reached the maternal circulation or amniotic fluid, amniotic fluid was sampled at 10, 60 and 180 minutes and maternal blood was sampled at 180 minutes.

Plasma was separated from whole blood, as described in Chapter 2. All plasma and amniotic samples were analysed within 24 hours of their collection. The disappearance of radioactivity from circulation was measured by counting duplicate 50 µl aliquots of foetal plasma in a gamma counter. Small samples of infused ^{125}I-IGF-I were also counted to enable calculation of foetal input radioactivity. The circulating forms of ^{125}I-IGF-I were characterised in plasma samples by size exclusion chromatography, as described in Chapter 2. Changes in the circulating forms of ^{125}I-IGF-I was determined in all plasma samples from the 12 minute and 180 minute animals. The radioactive content in duplicate 50 µl aliquots of maternal plasma and amniotic fluid was measured in a gamma counter.

Following slaughter, the hind limbs (femur and tibia) and fore limbs (humerus and radius) were dissected from each animal. Each bone was split longitudinally and either frozen for determination of radioactive content or fixed for histological analyses.

The radioactive content of bone fragments (frozen and fixed tissue) was measured in a gamma counter. Approximately 1 g of frozen tissue was analysed for each bone. Measurements were carried out on five epiphyses chosen randomly from eight frozen epiphyses collected for each animal. The eight epiphyses were from the proximal femur, distal tibia, proximal humerus and distal radius from the left and right limbs. Additionally, one bone that had been processed for histology (fixed and decalcified) from each animal was analysed. In these bones, the radioactive content of 0.2 to 1 g of tissue microdissected from the growth plate area, diaphysis and secondary ossification centre was measured.

The cellular localisation of radioactivity was determined in all bones from the 12 minute and 180 minute animals. Decalcified sections (7μm and 20 μm) from all animals were exposed to X-ray film for a maximum of 10 days, followed by exposure to liquid emulsion for a maximum of 80 days.

To investigate whether radioactivity and IGF-I were co-localised, sections were analysed by immunohistochemistry to detect IGF-I, prior to coating them with liquid emulsion for autoradiography, (described in Chapter 2). Bone sections representing the proximal femur from each animal were analysed. Sections were visually assessed and the results summarised for the 12 minute and 180 minute animals. The appropriate controls for immunohistochemistry were carried out, as already described (Chapters 3 and 4).

Reference samples of liver, kidney and skeletal muscle were also collected. The radioactive content of these tissues from one 12 minute and one 180 minute animal was measured in a similar way to bone. Approximately 1 g of frozen tissue from two different areas for each tissue were analysed in a gamma counter. Additionally, the radioactive content of three samples of skeletal muscle from each animal (surrounding bone processed for histological

analyses) were measured and compared to frozen tissue. Liver and skeletal muscle were sectioned at 7 μm and 20 μm and exposed to X-ray film and liquid emulsion alongside bone sections.

6.3 RESULTS

6.3.1 Distribution of Radioactivity in Tissues

The radioactive content of bone was similar at 12 minutes and 180 minutes following infusion of ^{125}I-IGF-I (Figure 6.1). This was observed with and without correction for differences in the plasma concentration of radioactivity between animals. Concentrations of radioactivity in plasma 1 minute after infusion of ^{125}I-IGF-I were used to correct for these differences. Correcting in this way adjusted for difference in the infused radioactivity (52 - 59 μCi) and variation in the total plasma volume between animals.

Compared to other tissues from the same animal, the radioactive content per weight of tissue was higher in bone than in skeletal muscle (Figure 6.1). However, levels were much lower in bone than in liver or kidney. While levels of radioactivity appeared to increase between 12 and 180 minutes following ^{125}I-IGF-I infusion in kidney, there was no difference in the content of radioactivity between samples of bone, skeletal muscle or liver collected at 12 or 180 minutes (Figure 6.1). However, as discussed below comparisons between tissues based on total radioactivity per gram of tissue do not necessarily reflect which tissues are the major targets of circulating ^{125}I-IGF-I.

Results representing total radioactivity in each tissue includes radioactivity from entrapped blood and radioactivity taken up into the tissue. Therefore the differences found in radioactive content between tissues may reflect differences between blood content and not differential uptake of ^{125}I-IGF-I into the tissue itself. Results were unable to be corrected for the component of radioactivity due to entrapped blood for practical reasons.

Figure 6.1 (a, b, c, d)

Radioactive content of plasma, bone measured in calcified and decalcified samples and other tissues from animals 12 minutes and 180 minutes following ^{125}I-IGF-I infusion. All measurements were corrected for ^{125}I decay and the relative level of radioactivity at 1 minute (to correct for variation in infused radioactivity and plasma volume between animals). No correction for entrapped blood was made for any tissue. Where appropriate, values are expressed as mean ± S.E.M for all animals at each timepoint.

6.1a

Radioactive content (cpm per 50 µl plasma) in plasma from animals sampled at 12 minutes (n=5) and animals sampled at 180 minutes (n=3) following ^{125}I-IGF-I infusion . Duplicate samples were measured.

6.1b

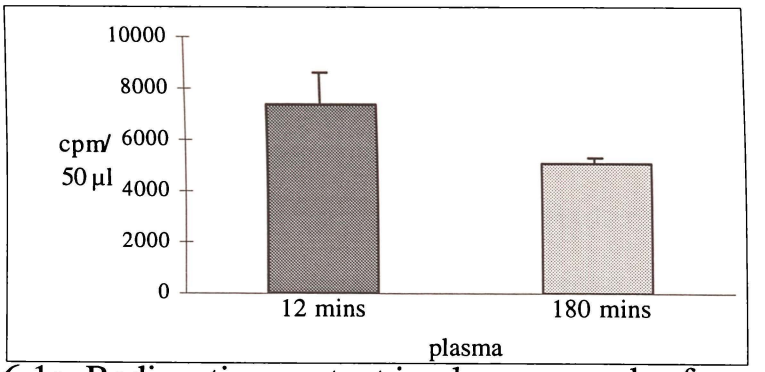
Radioactive content (cpm per g tissue) in calcified bone from animals sampled at 12 minutes (n=2) and animals sampled at 180 minutes (n=3) following ^{125}I-IGF-I infusion. Random samples of calcified bone from the epiphyses (n=5) were measured for each animal.

6.2c

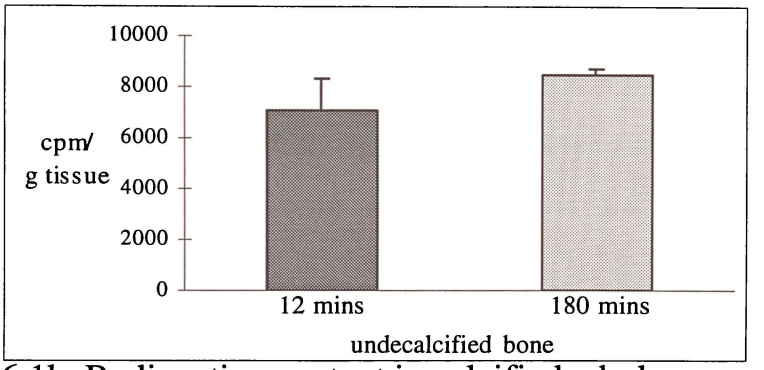
Radioactive content in different regions of decalcified bone. One random bone from animals sampled at 12 minutes (n=2) and animals sampled at 180 minutes (n=3) following ^{125}I-IGF-I infusion was dissected into three areas. The radioactive content of the secondary ossification centre, growth plate, and diaphysis were measured.

6.3d (reference tissues)

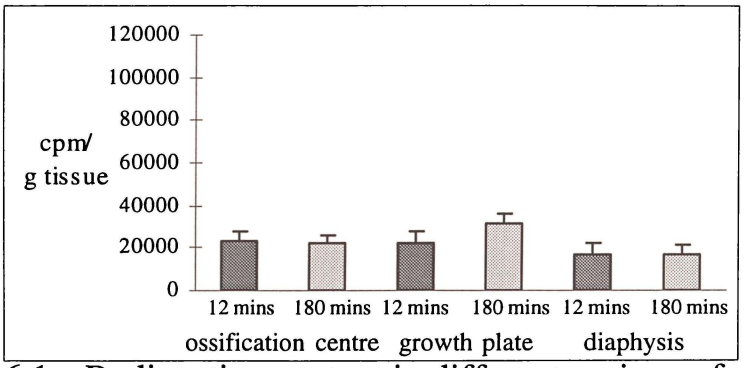
Radioactive content in skeletal muscle, liver and kidney samples collected from one animal at 12 minutes and 1 animal at 180 minutes following ^{125}I-IGF-I infusion. Each value represents the mean of 2 samples.



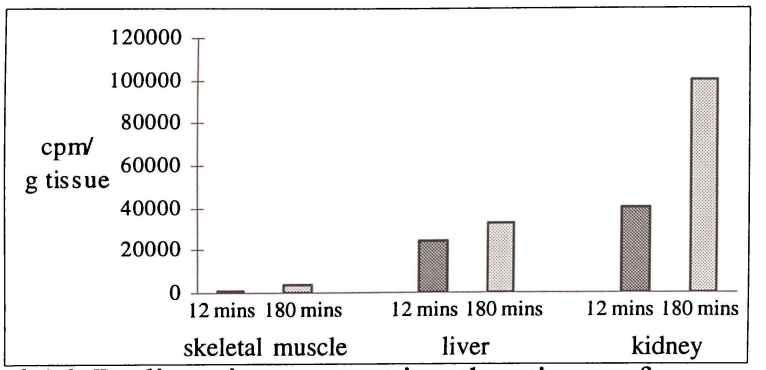
6.1a Radioactive content in plasma samples from animals 12 and 180 minutes following ^{125}I-IGF-I infusion



6.1b Radioactive content in calcified whole bone fragments from animals 12 and 180 minutes following ^{125}I-IGF-I infusion



6.1c Radioactive content in different regions of decalcified bone from animals at 12 and 180 minutes following ^{125}I-IGF-I infusion



6.1d Radioactive content in other tissues from one animal at 12 minutes and one animal at 180 minutes following ^{125}I-IGF-I infusion

In postnatal sheep, tissue blood content was reported to range from 6% in liver to 0.8% in skeletal muscle, which could account for some of the differences observed in radioactive content between tissues in the present study (Hodgkinson, 1991a). Furthermore, radioactivity was not detected at the cellular level in liver or skeletal muscle from 12 or 180 minute animals, indicating that total radioactivity measured in these tissues was likely to be due to entrapped blood, which was largely lost during histological processing (Figure 6.2). In contrast, radioactivity was detectable not only at sites where blood was located, but also in extravascular sites within bone (Figures 6.2, 6.3 and Table 6.1).

Another difficulty in comparing the content of total radioactivity between tissues and using it to assess which tissues are the more likely targets for ^{125}I-IGF-I is that individual tissue weights per unit area are not equivalent. Mineralised compounds in bone result in bone having a much higher weight per unit area compared to other tissues. Therefore, comparing on a weight basis underestimates the radioactive content of bone compared to other tissues. When the radioactive content of decalcified bone was compared with frozen calcified bone on a weight basis, decalcified bone overall displayed a much higher content of radioactivity than calcified bone per gram of tissue (Figure 6.1).

It was not possible to estimate the proportion of intact and degraded radioligand retained in each tissue. This was due to the low amount of radioactivity in these tissues and technical difficulties in adequately homogenising bone. However, it is unlikely that degradative products such as ^{125}I will contribute significantly to the radioactivity detected in these tissues. Not only was tracer integrity in plasma, as judged by size exclusion chromatography found to be high (see below), but in a similar study to this one, Lord *et al.* (1994) found negligible degradation products for ^{125}I-IGF-I in intestine perfused with ^{125}I-IGF-I, ^{125}I-des(1-3)IGF-I and ^{125}I-LR^{3}IGF-I for a period of 45 minutes. Furthermore, the relative content of radioactivity in liver, kidney and skeletal muscle in the present study was similar to the results of Ballard *et al.* (1991) and Cascieri *et al.* (1988) where intact ^{125}I-IGF-I was measured in these tissues at 10 minutes, 15 minutes or 2 hours following infusion.

	Immunoreactive IGF-I		Radioactivity (^{125}I-IGF-I)	
	12 minute animals	**180 minute animals**	**12 minute animals**	**180 minute animals**
perichondrium (chondrogenic cells)	+	++	–	–
reserve zone (chondrocytes)	+	+	–	–
proliferative zone (chondrocytes)	–	–	–	–
hypertrophic zone (chondrocytes)	+++	+++	++++ [(a)]	++++ [(a)]
cartilage matrix	–	–	++++ [(b)]	++++ [(b)]
periosteum (osteogenic cells)	+++	+++	–	–
bone cavity (osteogenic cells)	+	+	–	–
osteoblasts [(c)]	++++	++++	–	–
osteocytes	+++	++	–	–
bone matrix	+++	+++	–	–
metaphysis	++++	++++	++++	++++
vascular canals (capillary lumen)	+++	+++	++++	++++
vascular canals (connective tissue)	+++	+++	++++	++++
muscle fibres	++++	++++	–	–

Table 6.1

Summary of the localisation and relative abundance of radioactivity and immunoreactive IGF-I in different cells and regions of bone following infusion of ^{125}IGF-I. All bones and animals from the foetal infusion study were analysed. Bones were collected either 12 minutes or 180 minutes following infusion of ^{125}IGF-I.

Grain density and specific immunostaining were assessed on a scale using arbitrary units from high (++++) to low (+); (-) indicates an absence of radioactivity or immunoreactivity.

(a) lower two layers of hypertrophic cells adjacent to the metaphysis

(b) cartilage matrix at bone/cartilage boundaries

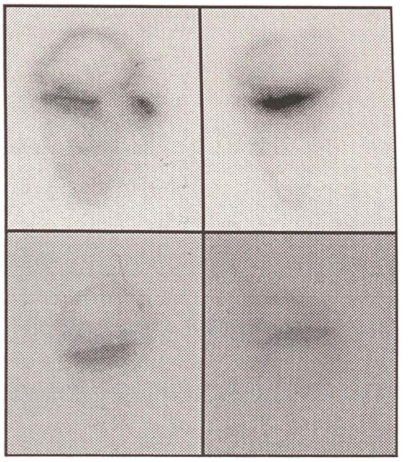
(c) results represent the majority of osteoblasts at all locations in bone

Figure 6.2

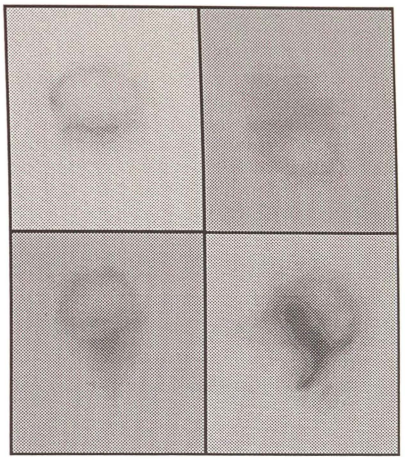
Representative bone sections from five animals infused with ^{125}I-IGF-I (~55 μCi; ~430 ng IGF-I). Bones were collected either 12 minutes or 180 minutes following infusion. Sectioning was at 20 μm and exposure to X-ray film was for 7 to 12 days.

Sections of liver and skeletal muscle collected from one animal 12 minutes and one animal 180 minutes following ^{125}I-IGF-I infusion are presented for comparison.

BONE
(epiphyses)

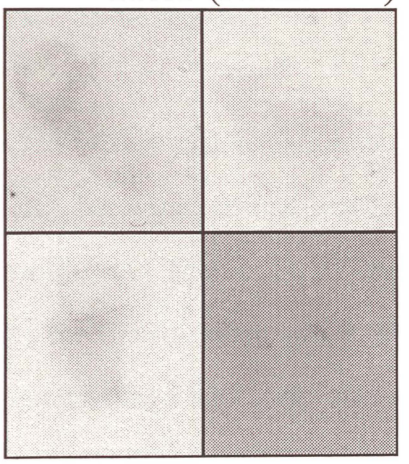


12 minutes (Animal 1)

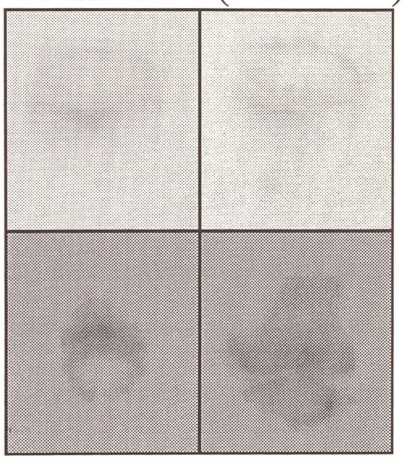


180 minutes (Animal 2)

BONE
(epiphyses)



12 minutes (Animal 3)



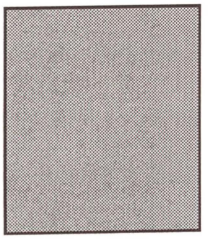
180 minutes (Animal 4)

BONE
(epiphyses)

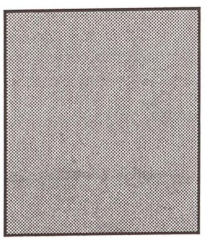


180 minutes (Animal 5)

SKELETAL
MUSCLE

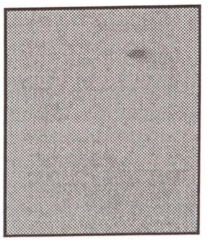


12 minutes
(Animal 3)

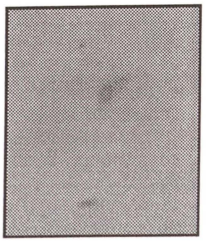


180 minutes
(Animal 5)

LIVER



12 minutes
(Animal 3)



180 minutes
(Animal 5)

6.3.2 Localisation of Radioactivity in Bone

Although the pattern of localisation was similar in animals sacrificed 12 minutes following ^{125}I-IGF-I infusion and 180 minutes following ^{125}I-IGF-I infusion, there was variation in the level of radioactivity between animals that reflected differences in the concentration of ^{125}I-IGF-I in plasma between animals (Figure 6.2). In bone sections from some animals the levels of radioactivity were too low to be detected.

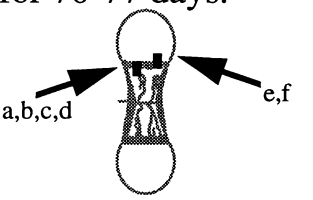
Radioactivity was restricted to two major histological areas within all bone sections. These were located at the boundary between cartilage and bone and within vascular canals embedded in the growth plate (Figure 6.3 and Table 6.1). Overall, both areas contained similar levels of radioactivity as judged by the density of autoradiographic grains (Figure 6.3 and Table 6.1). At the boundaries between cartilage and bone, radioactivity was restricted to a thin band of approximately 10 cells in width (Figure 6.2). Bands of radioactivity were clearly visible on X-ray film where they defined the perimeter of the secondary centre of ossification and the boundary between the epiphyseal growth plate and the metaphysis (Figure 6.2). The density of autoradiographic grains steeply declined to background levels at the edges of the band. The fall in the level of radioactivity outside the band was not defined by an obvious cellular boundary either in the hypertrophic zone or metaphysis, but appeared to occur at a set distance into these zones (Figure 6.3). At the level of the light microscope, the band of radioactivity extended approximately 15 μm (1 to 2 cells) into the lower hypertrophic cells of the growth plate and approximately 10 μm (5 to 10 cells) into the metaphysis (Figure 6.3).

Within the band, radioactivity was located both cellularly and extracellularly (Figure 6.3). The level of radioactivity was similar in cartilage extracellular matrix and hypertrophic cells. Similarly, radioactivity in the metaphysis was located at similar levels within cells, intercellular spaces and cartilage spicules extending into bone (Figure 6.3).

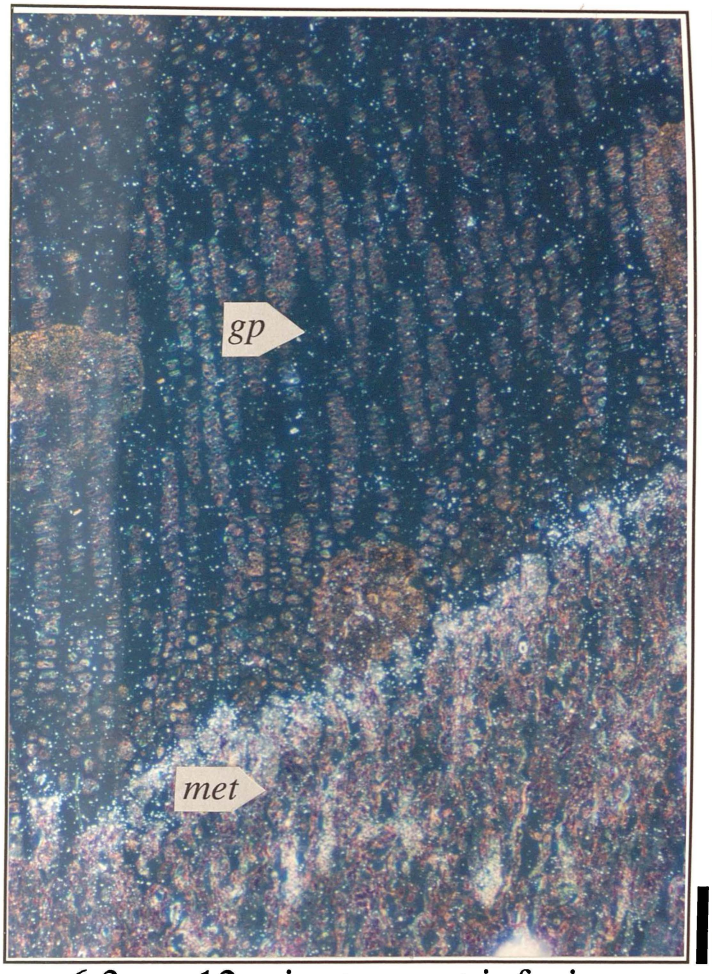
Figure 6.3 (a, b, c, d, e, f)

Bone sections from the proximal femur of animals infused with ^{125}I-IGF-I (~55 μCi; ~430 ng IGF-I). Bones were collected either 12 minutes following infusion of ^{125}I-IGF-I (a, c and e) or 180 minutes following infusion (b, d and f), processed and analysed by autoradiography. Radioactivity is visualised by dark field (white grains; a and b) or light field (black grains; c, d, e and f). Sectioning was at 7 μm. Exposure to emulsion was for 70-77 days.

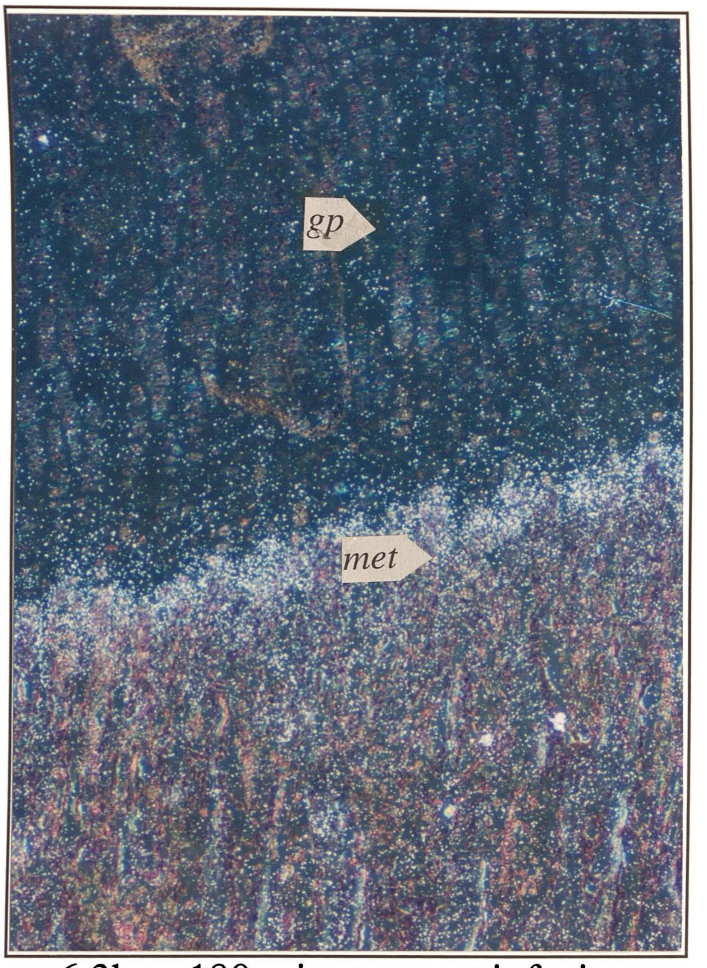
Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)



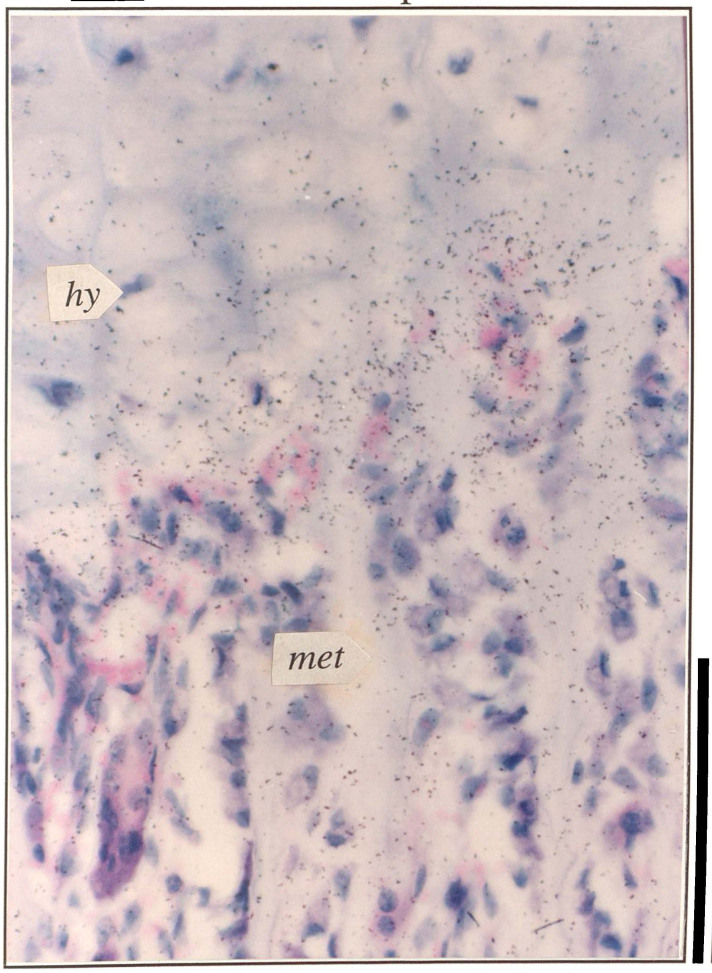
gp, growth plate; *met*, metaphysis; *res*, reserve chondrocyte; *hy*, hypertrophic chondrocyte; *c*, connective tissue; *vc*, vascular canal; *cl*, capillary lumen
Vertical Bar = 100 μm



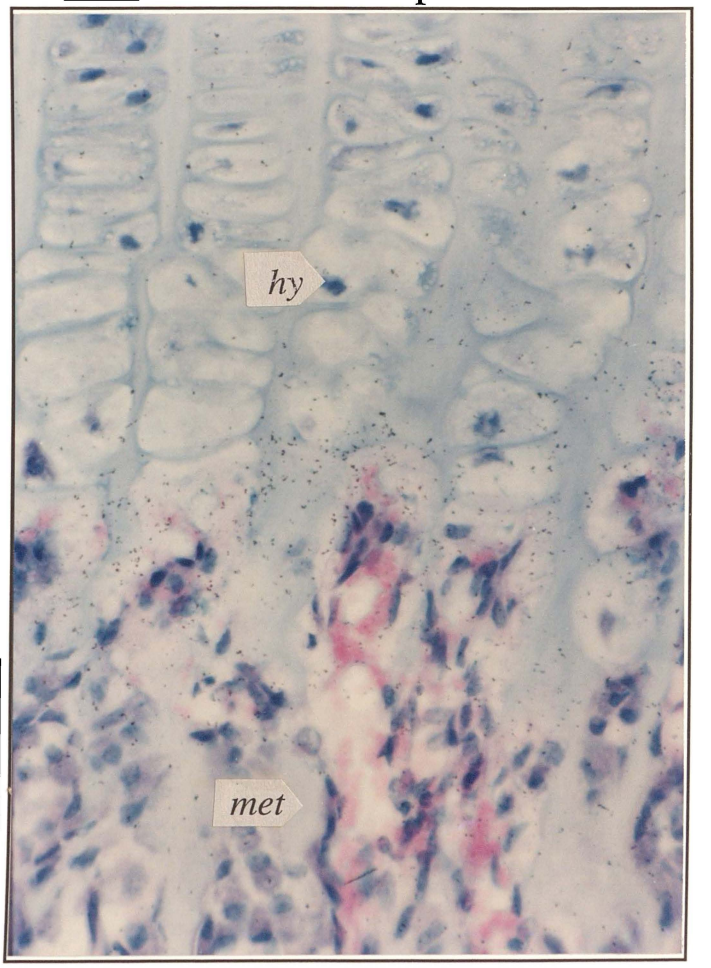
6.3a 12 minutes post infusion



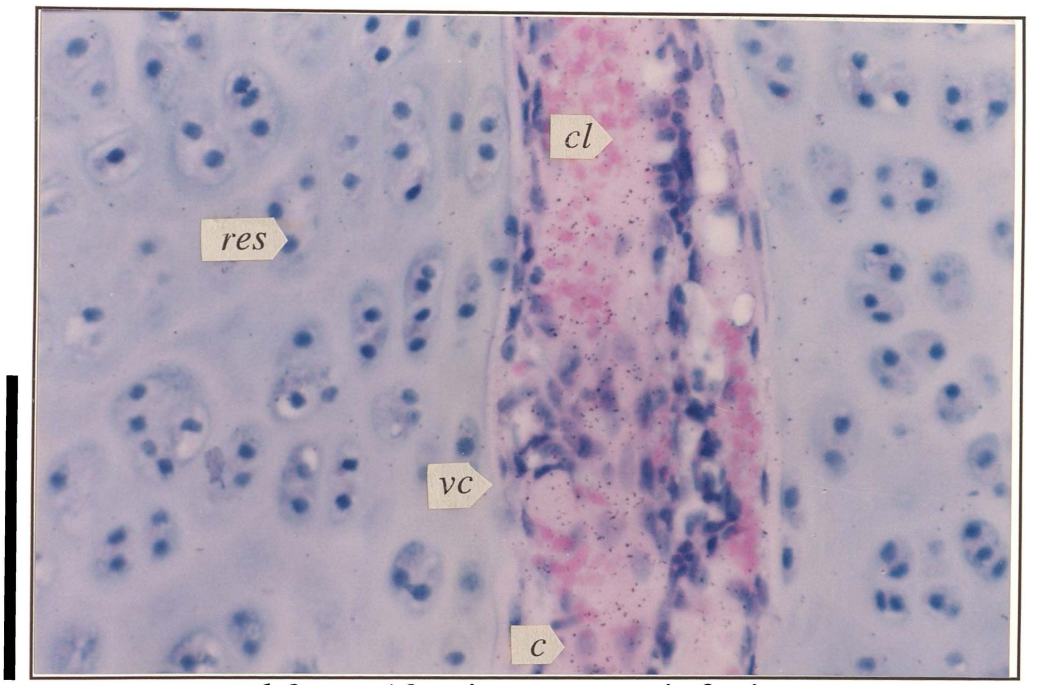
6.3b 180 minutes post infusion



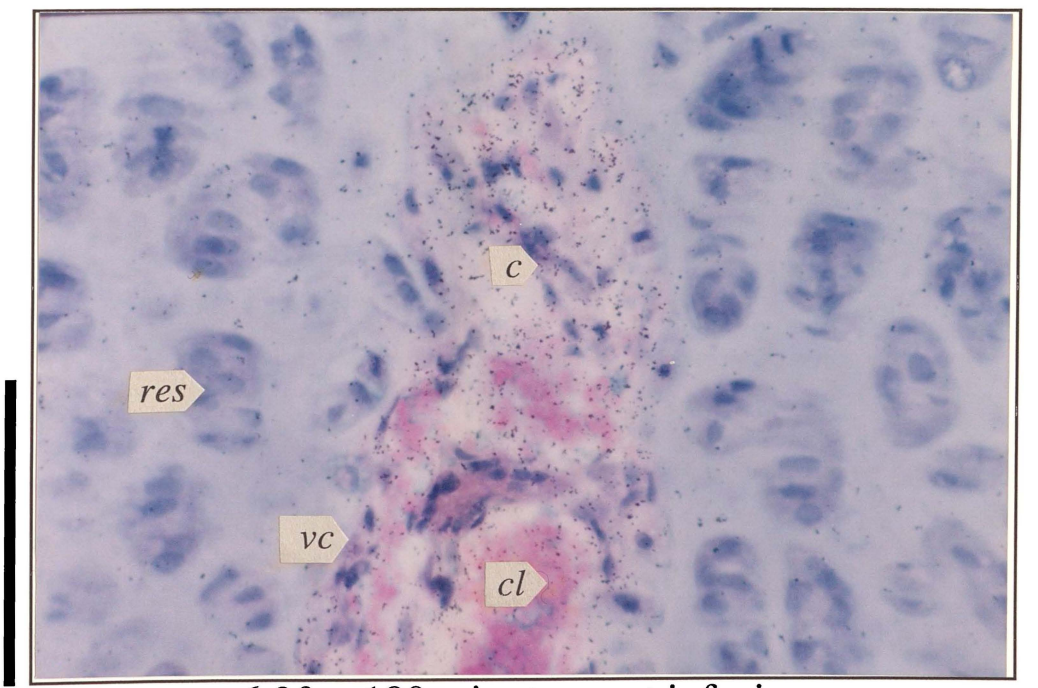
6.3c 12 minutes post infusion



6.3d 180 minutes post infusion



6.3e 12 minutes post infusion



6.3f 180 minutes post infusion

Within vascular canals embedded in the growth plate, radioactivity was associated not only with blood cells in capillary lumen, but also in connective tissue surrounding these capillaries (Figure 6.3). No radioactivity was observed in any section in cartilage tissue adjacent to the vascular canals (Figure 6.3). This was found regardless of which zone in the growth plate the vascular canal was located.

6.3.3 Co-localisation of Radioactivity and Immunoreactive IGF-I

Immunoreactive IGF-I was localised to cells that also contained radioactivity (Table 6.1 and Figure 6.4). These included the lower hypertrophic cells of the growth plate and cells in the metaphysis at the border between bone and cartilage. Connective tissue located in vascular canals and blood within the capillary lumen also co-localised both immunoreactive IGF-I and radioactivity (Figure 6.4). Additionally, there were areas within each bone section that contained radioactivity and no immunoreactive IGF-I and areas where there was no radioactivity but immunoreactive IGF-I (Table 6.1 and Figure 6.4).

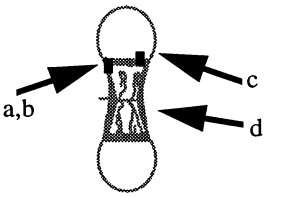
Cartilage extracellular matrix extending approximately 15 μm into the hypertrophic zone of the growth plate and cartilage spicules located in the first 10 μm of the metaphysis contained radioactivity, but did not stain for IGF-I (Figure 6.4). As discussed in Chapter 4, immunoreactivity for IGF-I was absent in cartilage matrix in all bone sections analysed. This is in agreement with previous reports where positive staining has been found over cartilage cells but not cartilage matrix (Middleton and Tyler, 1992; Hansson *et al.*, 1988; Nilsson *et al.*, 1986). A failure to detect IGF-I by immunohistochemistry could be due to IGF-I antibody binding sites being masked by IGF binding proteins and extracellular matrix components which are known to form complexes with IGF-I in the matrix (Hodgkinson *et al.*, 1994; Bautista *et al.*, 1991).

Positive staining for IGF-I was also localised to areas where radioactivity was not detectable (Table 6.1 and Figure 6.4). The distribution was similar to that which was previously found in bone sections from animals at a similar age (Chapter 4 and Table 4.1). Immunoreactive IGF-I

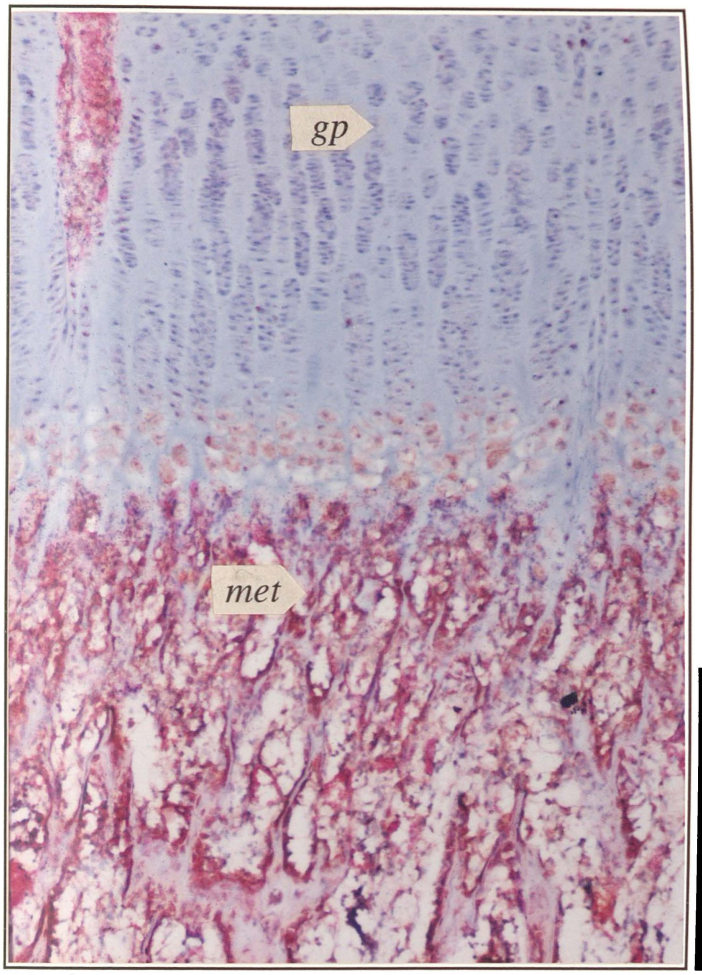
Figure 6.4 (a, b, c, d)

Bone sections from the proximal femur of animals infused with ^{125}I-IGF-I (~55 μCi; ~430 ng IGF-I). Bones were collected 180 minutes following infusion, processed, analysed for IGF-I by immunohistochemistry followed by autoradiography to determine the localisation of radioactivity. Sectioning was at 7 μm. Exposure to emulsion was for 70 days. Immunoreactive IGF-I appears as brown staining while black grains indicate the localisation of radioactivity.

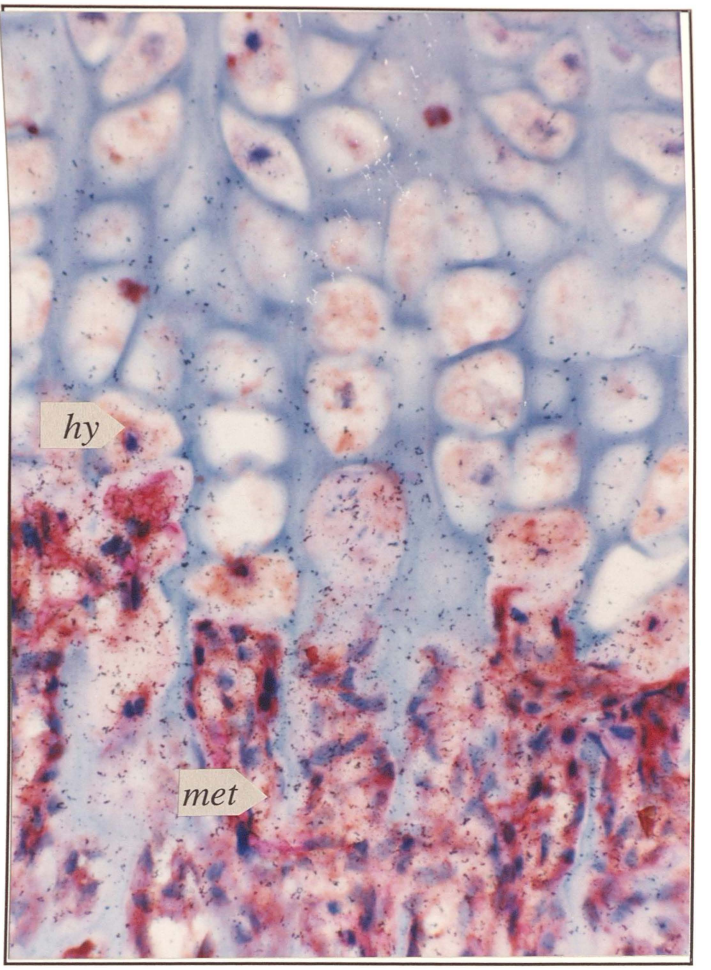
Photographs are orientated in the longitudinal plane:
(a view through the epiphyseal growth plate)



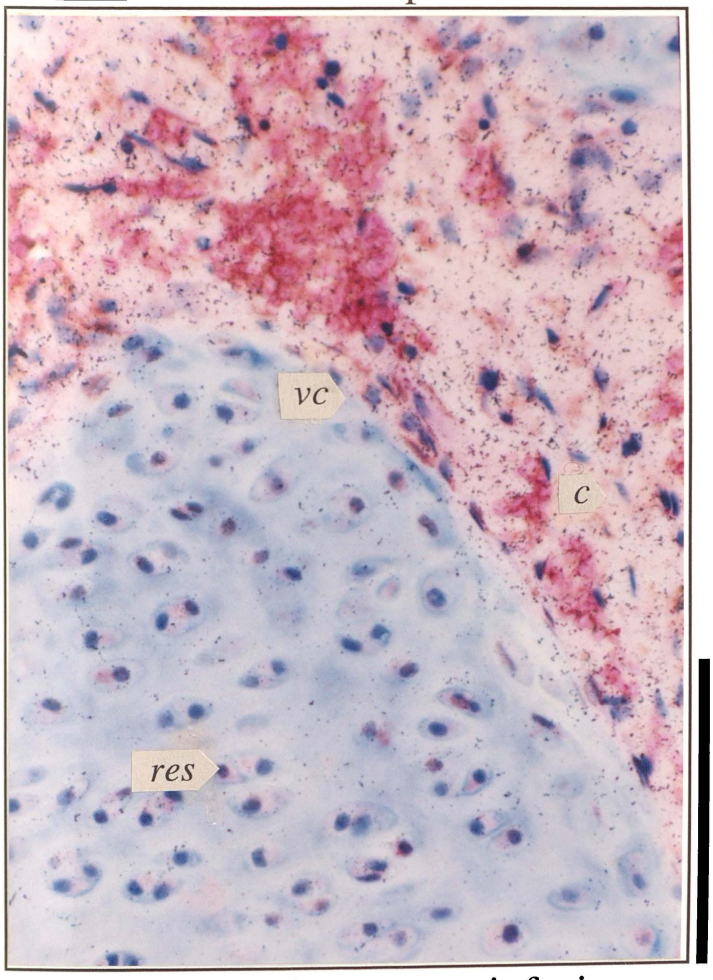
gp, growth plate; *met*, metaphysis; *hy*, hypertrophic chondrocyte; *c*, connective tissue; *vc*, vascular canal; *res*, reserve chondrocyte; *osteo*, osteogenic cell; *ost*, osteoblast; *ostc*, osteocyte; *bm*, bone matrix
Vertical Bar = 100 μm



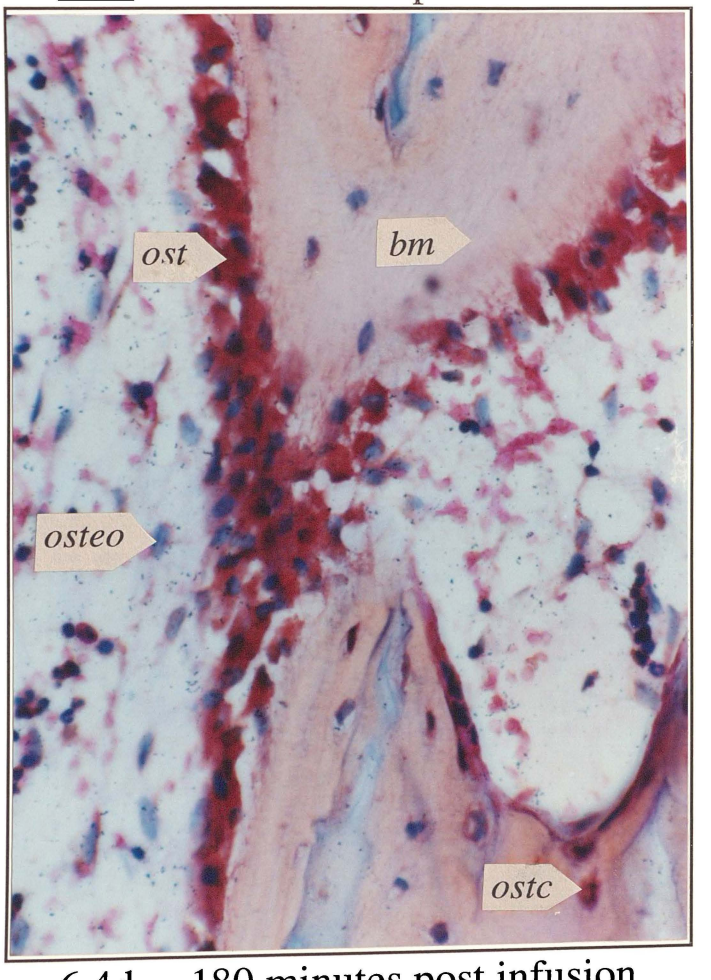
6.4a 180 minutes post infusion



6.4b 180 minutes post infusion



6.4c 180 minutes post infusion



6.4d 180 minutes post infusion

was highest in osteoblasts, with positive staining also detectable in osteogenic cells residing in the periosteum, osteocytes and bone matrix. In contrast to the pattern that was found for animals from this age (140 days gestation) in the foetal ontogeny study (Chapter 4), much lower staining in the proliferative and reserve cartilage zones was observed in the study described in this chapter. The pattern of staining was similar to that which was found in younger animals (50 to 103 days gestation) from the foetal ontogeny study (Chapter 4).

6.3.4 Profiles of ^{125}I-IGF-I in Plasma

Following ^{125}I-IGF-I infusion there was an initial rapid decrease in the levels of ^{125}I-IGF-I in plasma. This was followed by a slower decline in ^{125}I-IGF-I to reach levels at 180 minutes that were between 36% and 55% of those observed at 1 minute (Figure 6.5).

The circulating forms of ^{125}I-IGF-I present in plasma following ^{125}I-IGF-I infusion were similar for all animals as determined by size exclusion chromatography (Figure 6.6). Typical profiles observed during the 180 minute period following ^{125}I-IGF-I infusion are presented in Figure 6.6 for two representative animals. In plasma samples taken 1 minute after ^{125}I-IGF-I infusion, either two (3 animals) or three (2 animals) peaks of radioactivity were observed. These corresponded to 'free' ^{125}I-IGF-I (with a peak height at fraction 30), a 40-50 kDa pool of IGF BP complexed to ^{125}I-IGF-I (with a peak height at fraction 23) and a 150 kDa pool of IGF BP complexed to ^{125}I-IGF-I (with a peak height at fraction 15). The identity of these ^{125}I-IGF-I complexes has previously been characterised (Hodgkinson, 1991a). Furthermore, when ^{125}I-IGF-I was incubated with sheep serum and subjected to size exclusion chromatography, a profile containing two peaks eluting at fraction 23 and 15, corresponding to the 40-50 kDa IGF BP complexes and 150 kDa complex, respectively, were observed.

'Free' ^{125}I-IGF-I declined rapidly after infusion, with detection of its presence in only two of the five animals 1 minute following ^{125}I-IGF-I infusion (Figure 6.6). By 2 minutes, 'free' ^{125}I-IGF-I was no longer detected in plasma samples from any animal (Figure 6.6).

Figure 6.5
CLEARANCE OF RADIOACTIVITY IN PLASMA FOLLOWING INFUSION OF ^{125}I-IGF-I

The change in total radioactivity or protein bound radioactivity in plasma following ^{125}I-IGF-I infusion. Typical profiles of two animals over the 180 minute period are shown. Total radioactivity was determined in duplicate 100 µl samples of plasma. Protein bound radioactivity was determined as the total radioactivity measured in fractions 10 to 43 by size exclusion chromatography. Arrows indicate tissue collection times.

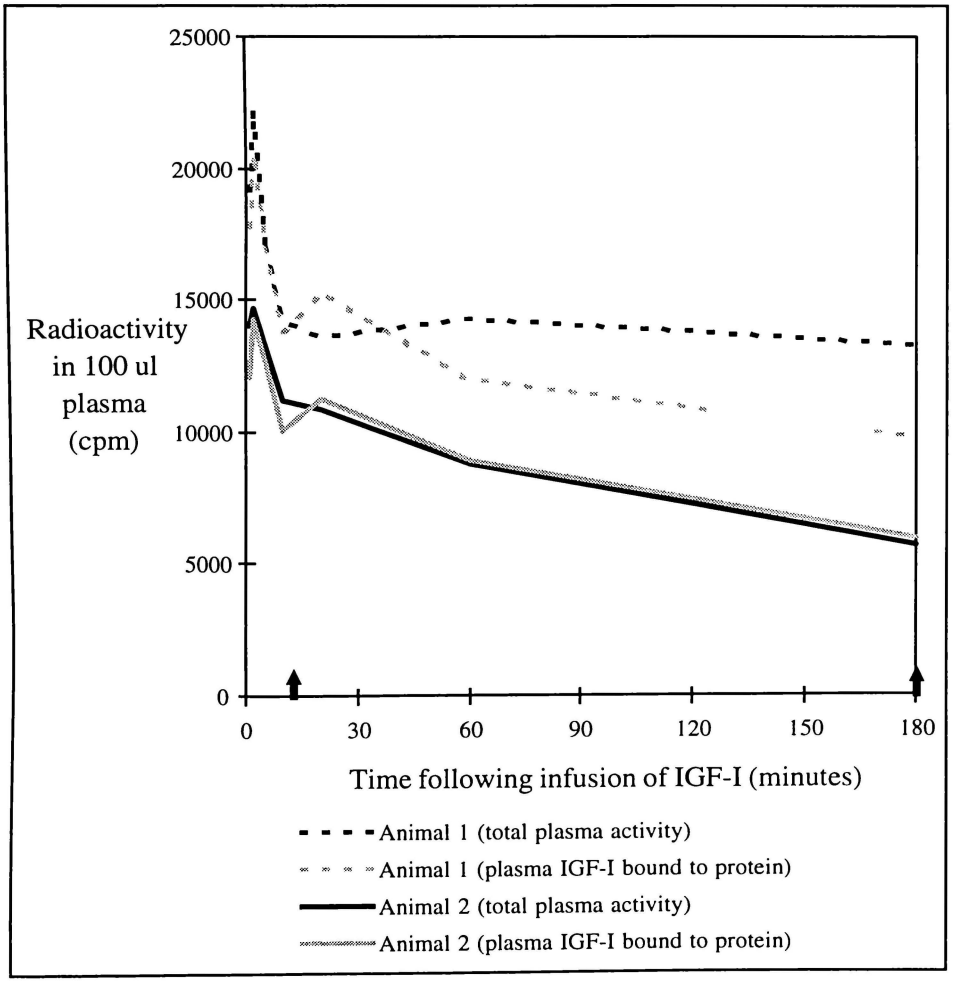
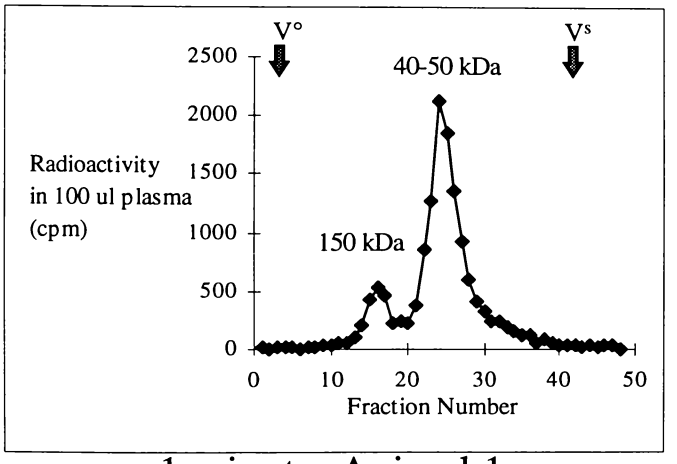
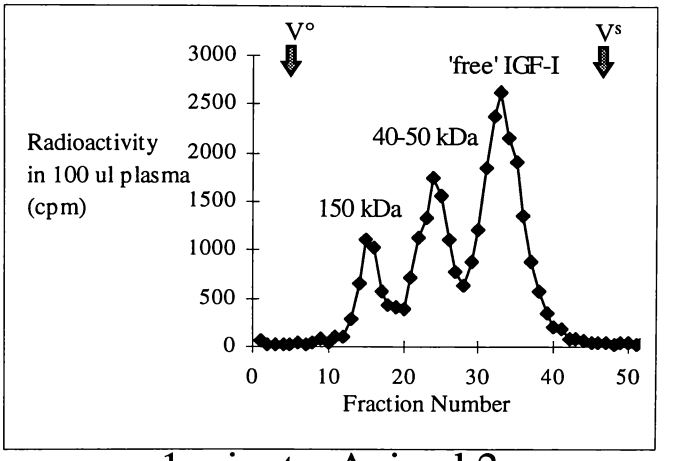


Figure 6.6
PROFILES OBTAINED BY SIZE EXCLUSION CHROMATOGRAPHY

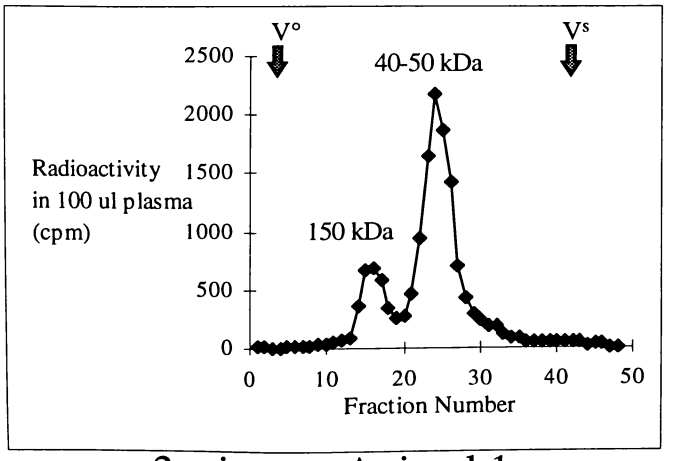
The different forms of ^{125}I-IGF-I circulating in plasma following ^{125}I-IGF-I infusion. Size exclusion chromatography profiles of two representative animals over the 180 minute period are shown. The three peaks correspond to the expected elution positions of 'free' ^{125}I-IGF-I , ^{125}I-IGF-I bound to a 40-50 kDa pool of IGF BP and ^{125}I-IGF-I bound to a 150 kDa pool of IGF BP, as indicated. The void and salt volumes of the column are indicated by V° and V^s, respectively.



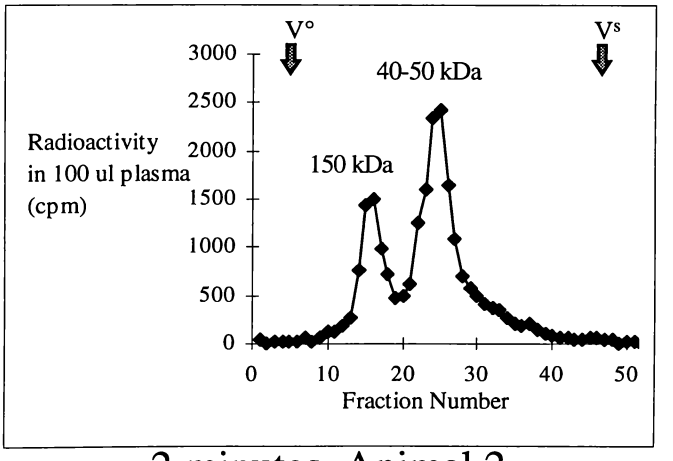
1 minute, Animal 1



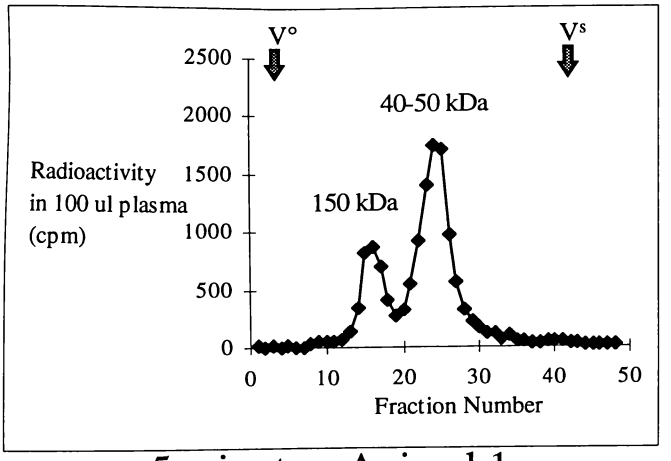
1 minute, Animal 2



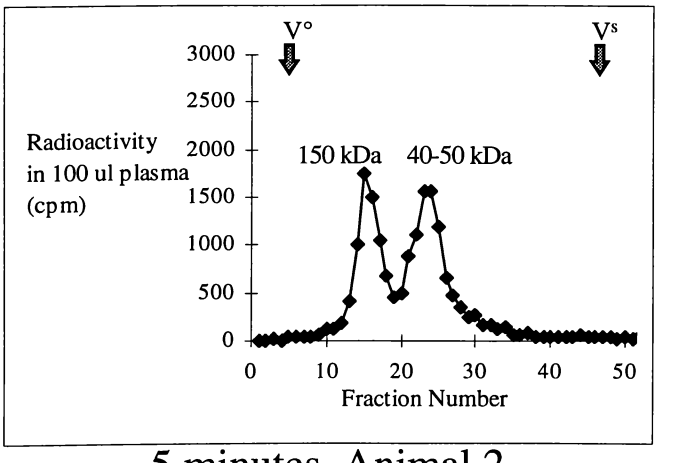
2 minutes, Animal 1



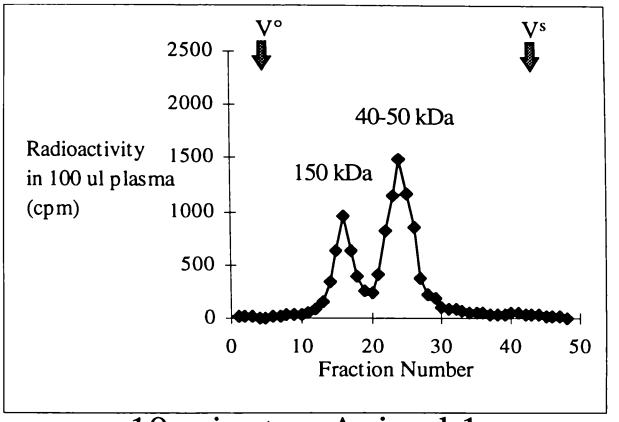
2 minutes, Animal 2



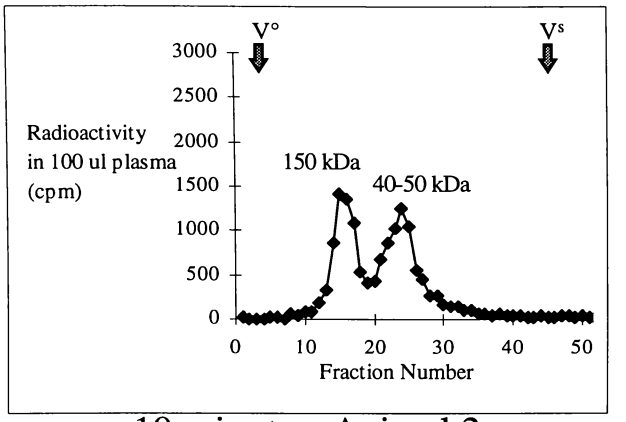
5 minutes, Animal 1



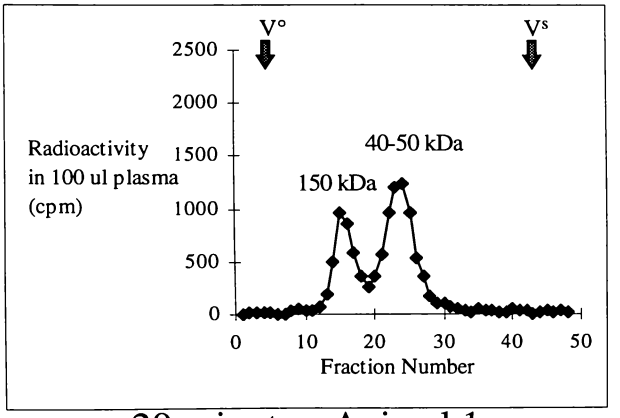
5 minutes, Animal 2



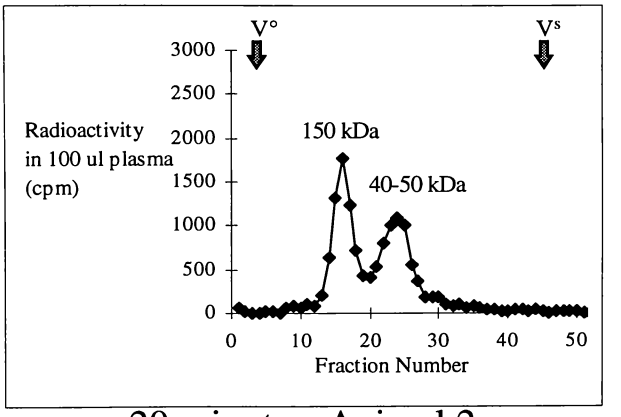
10 minutes, Animal 1



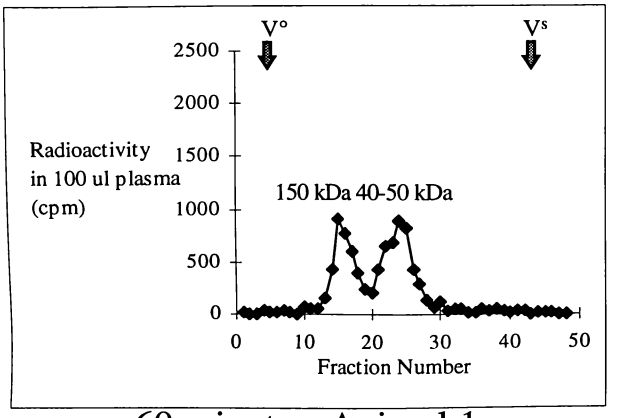
10 minutes, Animal 2



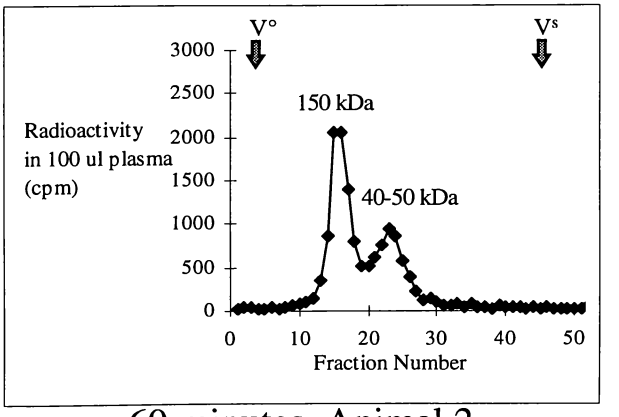
20 minutes, Animal 1



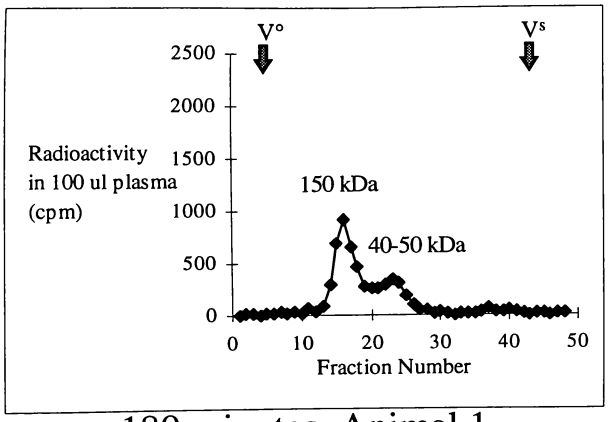
20 minutes, Animal 2



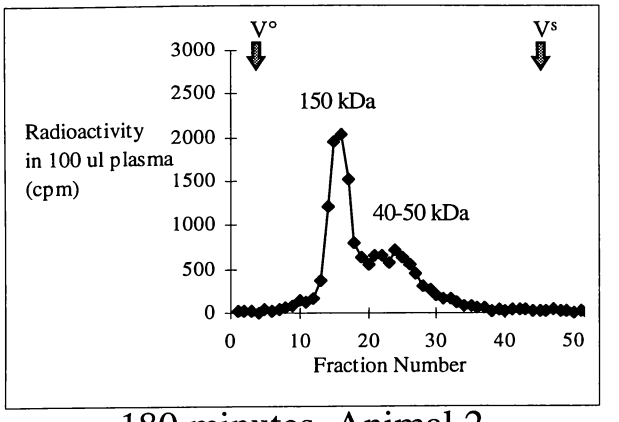
60 minutes, Animal 1



60 minutes, Animal 2



180 minutes, Animal 1



180 minutes, Animal 2

The two peaks corresponding to the 40-50 kDa and 150 kDa complexes were present in all samples (Figure 6.6). The height of the 40-50 kDa peak was highest at 1 minute, after which it gradually declined to be barely detectable by 180 minutes. In contrast, the height of the 150 kDa peak was lowest at 1 minute. It increased gradually to reach its maximum 20 minutes following ^{125}I-IGF-I infusion after which it remained at a similar level for the rest of the sampling period.

Tracer integrity was assessed by comparing radioactivity eluting in protein bound fractions (as judged by size exclusion chromatography) to total radioactivity for each plasma sample. There appeared to be only negligible tracer degradation at the later sampling points in some animals (Figure 6.5). Additionally, no radioactivity was found at the void and salt volumes of the column and no evidence of intermediate degradative products were observed.

6.3.5 Radioactivity in Maternal Plasma and Amniotic Fluid

No radioactivity could be detected in maternal plasma samples from any animal (data not shown). Similarly, radioactivity was not found in amniotic fluid at any point following infusion of ^{125}I-IGF-I (data not shown). These results suggest that there was no transfer of ^{125}I-IGF-I from the foetus across the placenta into the maternal circulation and that ^{125}I-IGF-I was not excreted into amniotic fluid in significant amounts during the 180 minute period.

6.4 DISCUSSION

6.4.1 Uptake of Circulating IGF-I in Foetal Sheep Bone

Results from the present study support an endocrine role for IGF-I in foetal bone. However the relative importance and target cells for this mode of action in the foetal sheep requires further investigation. The areas where circulating IGF-I was located in bone in the present study are different to those areas traditionally thought to be capable of responding directly to endocrine stimulation.

Radioactivity taken up by bone following ^{125}I-IGF-I infusion into foetal sheep was distributed at the cellular level in two main areas of bone that reflected the independent blood supplies for the epiphysis and metaphysis/diaphysis in sheep (Levene, 1964). ^{125}I-IGF-I derived from the metaphyseal circulation was localised in a transverse band at the boundary between the growth plate and metaphysis. ^{125}I-IGF-I derived from the epiphyseal circulation was associated with connective tissue and capillary lumen within vascular canals located in various zones of the growth plate. The movement of IGF-I from the circulation into bone tissue suggests that bone is a potential target for the actions of circulating IGF-I.

An endocrine role for IGF-I has been reported in postnatal rats. An increase in IGF-I in the circulation of hypophysectomised or insulin-deficient diabetic rats stimulates bone growth parameters such as growth plate width, which is an indicator of long bone growth (Guler *et al.*, 1988; Scheiwiller *et al.*, 1986; Schlechter *et al.*, 1986; Schoenle *et al.*, 1985; Schoenle *et al.*, 1982) and accumulated bone growth (Glassock *et al.*, 1992; Guler *et al.*, 1988). Circulating IGF-I also stimulates osteoblast activity (Machwate *et al.*, 1994) and bone formation and resorption (Spencer *et al.*, 1991) in postnatal rats. In foetal sheep, the levels of circulating IGF-I correlate positively with foetal weight (Carr *et al.*, 1995). Although Carr *et al.* (1995) did not investigate bone weight or size, it is possible that this correlation also holds for long bone growth which would provide indirect evidence for an endocrine role for IGF-I in this tissue in the foetus.

It is generally believed that the target cells for these growth promoting actions of circulating IGF-I are chondrocytes located in the growth plate, since this is the area of bone responsible for longitudinal bone growth. In support of this, infusion of IGF-I into the circulation of hypophysectomised rats results in a significant increase in cell size and cell productivity in chondrocytes from all zones of the growth plate (Hunziker *et al.*, 1994). In cultured chondrocytes isolated from the growth plate of a range of species and stages of development, IGF-I stimulates cell proliferation and matrix production, which is further evidence that these cells are able to respond to IGF-I (Hill *et al.*, 1992a; Hill and De Sousa, 1990a; Ohlsson *et al.*,

1992a; Böhme *et al.*, 1992; Demarquay *et al.*, 1990; Lindahl *et al.*, 1987a; Lindahl *et al.*, 1987b; Hill, 1979).

Results from the present study show that circulating ^{125}I-IGF-I *in vivo* is unable to penetrate into those zones of the growth plate where maximal chondrocyte proliferation and matrix production occur. This is supported by the results of Schneiderman *et al.* (1995a) who found that IGF-I complexed to IGF binding proteins and to a lesser extent 'free' IGF-I are largely excluded from entering normal articular cartilage due to the barrier posed by glycosaminoglycans in the extracellular matrix (Maroudas, 1976). Furthermore, entry from the metaphyseal side of the growth plate is likely to be hindered by matrix calcification in the lower hypertrophic zone which is believed to prevent nutrient delivery and contribute to the subsequent demise of hypertrophic chondrocytes (Poole, 1991).

The inability to detect circulating IGF-I in the growth plate can be interpreted in two ways. Either circulating IGF-I has little impact on the overall actions attributable to IGF-I in the growth of normal foetal bone or effects of circulating IGF-I on chondrocytes in the growth plate are indirect via endocrine actions on cells outside the growth plate.

The endocrine actions described for IGF-I *in vivo* have generally only been reported in postnatal pituitary-deficient rats or people with Laron Syndrome treated with IGF-I, to either restore or exceed the normal circulating levels of IGF-I (Hunziker *et al.*, 1994; Laron and Klinger, 1994; Vaccarello *et al.*, 1993; Heinrichs *et al.*, 1993; Glassock *et al.*, 1992; Walker and McNicol, 1992; Laron *et al.*, 1992; Guler *et al.*, 1988; Schlechter *et al.*, 1986; Schoenle *et al.*, 1985; Schoenle *et al.*, 1982). The effects of IGF-I administration in these studies is small, and although significant, does not restore bone growth to normal.

In hypophysectomised rats, the addition of GH is much more potent at stimulating bone growth than IGF-I, suggesting that a paracrine or autocrine mode of action for IGF-I is potentially far more important than an endocrine mode of action in these animals (Hunziker *et al.*, 1994; Glassock *et al.*, 1992; Schoenle *et al.*, 1985). Hypophysectomised animals contain

low levels of circulating IGF-I and very low levels of growth hormone (GH). The addition of GH increases not only levels of circulating IGF-I (Mesiano *et al.*, 1989) but stimulates local production of IGF-I in the growth plate (Isgaard, 1992; Nilsson *et al.*, 1990; Isgaard *et al.*, 1988a; Isgaard *et al.*, 1988b). The large difference in potency between IGF-I and GH is therefore thought to be due to GH actions on the local production of IGF-I. This finding is supported by the results of Behringer *et al.* (1990) who found that GH deficient mice also transgenic for IGF-I display relatively normal growth. However, some studies have demonstrated that GH has direct effects in the growth plate which could account for some of these GH effects (Ohlsson *et al.*, 1992b; Green *et al.*, 1985). In support of a role for locally produced IGF-I, direct administration of IGF-I into the growth plate of hypophysectomised rats to mimic the actions of local IGF-I is able to stimulate an increase in growth plate width and an increase in longitudinal bone growth (Isgaard *et al.*, 1986; Russel and Spencer, 1985).

Overall, in Laron Syndrome and the GH deficient rat models, evidence suggests that there is an endocrine mode of action for IGF-I but it appears to be less important than an autocrine or paracrine mode of action. However, the relative importance of locally produced versus serum-derived IGF-I is difficult to assess.

In intact animals, circulating IGF-I appears to be even less effective than in hypophysectomised animals. Circulating IGF-I does not appear to have any significant effects on bone growth in intact rats or mice transgenic for IGF-I unless extremely high levels of IGF-I are achieved (Mathews *et al.*, 1988; Skottner *et al.*, 1987). It is possible that an endocrine mode of action for IGF-I only becomes important when there is insufficient local production of IGF-I in the growth plate, as is the case in Laron syndrome or GH-deficient rats, or if the cartilage has an increased requirement for IGF-I that cannot be met by an increase in local production.

In osteoarthritis, there appears to be an increase in the permeability of the matrix in articular cartilage as a result of a change in the composition of proteoglycans (Schneiderman *et al.*, 1995b). In normal articular cartilage, both the level of IGF-I able to penetrate into cartilage,

and the level of extractable IGF-I is likely to be far below that which is required to generate a minimal biological response in chondrocytes (Schneiderman *et al.*, 1995b; Luyten *et al.*, 1988; D'Ercole *et al.*, 1984). It is postulated that IGF-I reaches a level that is biologically important in osteoarthritis as a result of an increase in the permeability of the cartilage matrix to allow IGF-I circulating in its bound form to enter articular cartilage from the synovial fluid (Schneiderman *et al.*, 1995b).

The inability to detect IGF-I uptake into growth plates from normally growing foetal animals in the present study may be due to sufficient levels of IGF-I already being present in the growth plate and therefore only a small ongoing requirement for IGF-I from the circulation. Most IGF-I in growth plate cartilage could be accounted for by low, continuous local production coupled with storage in the extracellular matrix (Jones and Clemmons, 1995; Hodgkinson *et al.*, 1995a; Hodgkinson *et al.*, 1995b; Jones *et al.*, 1993). Circulating IGF-I may contribute to this pool of IGF-I but levels of uptake in normal foetal sheep may be too low to be detectable.

In the present study ^{125}I-IGF-I formed less than 1% of circulating IGF-I in these animals which may not have been sensitive enough to detect very low levels of IGF-I. The level of sensitivity could have been improved by increasing the contribution of IGF-I tracer to the total pool of IGF-I. While no radioactivity was detectable at the cellular level in skeletal muscle in the present study following injection of ^{125}I-IGF-I into the circulation, circulating IGF-I is detectable in fibres and connective tissue in skeletal muscle when high levels of N-met IGF-I are infused into the circulation of neonatal sheep for a period of 24 hours (Hodgkinson *et al.*, 1991b).

The presence of such low levels of total IGF-I in the growth plate of normal animals is predicted to be inadequate to account for the observed biological actions of IGF-I on chondrocytes. However, it is possible that the additive effects displayed by IGF-I with a range of other growth factors *in vitro* could be sufficient to achieve the results observed *in vivo*. IGF-I is more potent at stimulating the uptake of labelled amino acids into protein and

thymidine incorporation into DNA in the presence of bFGF, TGFβ and insulin in cultured chondrocytes (Trippel *et al.*, 1993; Hill *et al.*, 1992a; Hill and Logan, 1992b; Hill and De Sousa, 1990a).

It is not clear if cells located in the areas where ^{125}I-IGF-I was distributed represent target cells for the direct endocrine actions of IGF-I. It could be argued that ^{125}I-IGF-I was present in these areas of bone purely as a result of the particular capillary architecture at these locations, which suggests little selectivity in transcapillary transport. The points at which ^{125}I-IGF-I exited the circulation are characterised by capillary walls that are fenestrated and frequently discontinuous in all species studied (Rodríguez *et al.*, 1985; Howlett *et al.*, 1984; Howlett, 1980; Wilsman and Van Sickle, 1971). It is therefore likely that all of the circulating forms of IGF-I are able to exit the circulation in these areas, regardless of their size.

Cells located in the lower hypertrophic zone, upper metaphysis and vascular canal where ^{125}I-IGF-I was distributed, can all potentially respond to the actions of IGF-I. The area at the boundary between the growth plate and bone is characterised by cartilage calcification, bone formation, cartilage degradation and angiogenesis. Vascular canals are reported to not only play a role in nutrient delivery to the growth plate, but mesenchymal cells located in the connective tissue may serve as a stem cell population for interstitial growth within the growth plate (Wilsman and Van Sickle, 1971). *In vitro* studies have shown that IGF-I stimulates differentiation of osteoblasts (Canalis *et al.*, 1991; McCarthy *et al.*, 1989b; Hock *et al.*, 1988; Schmid *et al.*, 1984), formation and activation of osteoclasts (Hill *et al.*, 1995; Mochizuki *et al.*, 1992; Slootweg *et al.*, 1992) and angiogenesis (Bar *et al.*, 1994; Nakao-Hayashi *et al.*, 1992). In the coupling of processes in the metaphyseal area, IGF-I has been shown to stimulate chemotaxis of osteoclast progenitor cells as a result of an association with vascular endothelial cells (Formigli *et al.*, 1995).

Some of these actions could be sufficient to produce indirect effects on chondrocytes located in the growth plate which would result in the overall effects of IGF-I observed *in vivo*. Recently, a greater number of studies investigating the importance of vascularisation at the

epiphyseal-metaphyseal juncture to hypertrophic cartilage degradation and in the overall co-ordination of endochondral ossification have been undertaken (Alini *et al.*, 1996; Caplan, 1990; Caplan, 1988; Trueta, 1963).

IGF-I derived from the circulation was found to be co-localised to immunoreactive IGF-I in the present study. In addition, there were areas in bone that contained immunoreactive IGF-I but no ^{125}I-IGF-I. This suggests that locally produced IGF-I may target some cells in bone that are inaccessible to circulating IGF-I. A low level of IGF-I mRNA was also located in these areas (as described in the previous Chapter), which is likely to account for the presence of immunoreactive IGF-I in these areas. Paracrine or autocrine actions for IGF-I may therefore have a greater importance in some areas of bone than an endocrine mode of action.

6.4.2 Profile of ^{125}I-IGF-I in the Circulation in Relation to its Localisation in Bone

Following infusion, ^{125}I-IGF-I was immediately sequestered by binding proteins in a similar pattern to that which has been reported previously in the sheep and other species, which confirms the efficacy of ^{125}I-IGF-I used in the present study (Prosser *et al.*, 1992; Thissen *et al.*, 1992b; Ballard *et al.*, 1991; Bassett *et al.*, 1990; Francis *et al.*, 1990; Francis *et al.*, 1988; Hodgkinson *et al.*, 1987). Over time, the level of ^{125}I-IGF-I associated with the 40-50 kDa binding proteins declined to be almost absent 180 minutes following ^{125}I-IGF-I infusion, while ^{125}I-IGF-I associated with the 150 kDa binding protein showed an initial increase, following which levels remained high for the rest of the 180 minute period investigated.

Over the 180 minute period, levels of radioactivity in plasma declined to between 36% and 55% of those observed at 1 minute. This decrease was not due to movement of radioactivity outside the foetus into the maternal circulation or amniotic fluid. No radioactivity was detectable in these two fluids at 10, 60 or 180 minutes. Similarly, Bassett *et al.* (1990) did not detect intact IGF-I in an extensive number of fluids such as foetal urine, amniotic fluid, maternal uterine venous plasma and maternal systemic plasma (Bassett *et al.*, 1990). These results suggest that 45% to 65% of the radioactivity left the circulation and entered foetal

tissues during the 180 minute period. In the present study, it was found that some of this radioactivity was located in the kidney and liver. Although other tissues were not investigated in this study, Ballard *et al.* (1991) and Cascieri *et al.* (1988) report the uptake of ^{125}I-IGF-I into many tissues in the postnatal rat including kidney, liver, skin and the digestive tract.

Changes in the association of ^{125}I-IGF-I with the 40-50 or 150 kDa binding protein complexes over time did not influence the distribution of ^{125}I-IGF-I found in bone between the 12 minute and 180 minute sampling periods. These results suggest that all circulating forms of IGF-I were able to leave the circulation to a similar extent. It is unlikely that the radioactivity represented only 'free' IGF-I that entered bone in the first few minutes following infusion, since although between the 12 and 180 minute timepoints the pattern of localisation was identical, the lower hypertrophic cell layer present at 12 minutes would have been completely degraded by 180 minutes (Poole, 1991).

A recent study found that infusion into the artery supplying the intestine in lambs of variant IGF-I species with different affinities for the 40-50 kDa binding complexes results in a similar net transfer of IGF-I from blood into tissue indicating that the association of IGF-I with these binding proteins does not effect the transfer of IGF-I out of the circulation (Lord *et al.*, 1994). Similarly, perfusion of IGF BP-1, -2, -3 or -4 in isolated, beating hearts results in transcapillary movement of each IGF BP into connective tissue and cardiac muscle fibres within 1 minute of administration, suggesting that the 40-50 kDa binding proteins are readily able to cross the capillary boundary (Boes *et al.*, 1992; Bar *et al.*, 1992; Bar *et al.*, 1990).

Overall, results demonstrate that IGF-I from the circulation is able to enter bone, where it distributes in two specific areas associated with the growth plate, suggesting an endocrine mode of action for IGF-I in bone. However, the target cells for this mode of action are unclear. IGF-I may either have direct effects in the two areas where it was located and indirect effects in the growth plate, or direct effects in the growth plate as a result of a low level of accumulated uptake through the cartilage matrix. Local production appears to be important in those areas of bone that are inaccessible to IGF-I derived from the circulation.

CHAPTER 7

GENERAL DISCUSSION

Central to the growth and development of long bones, as with all other tissues, are the processes of cell division and differentiation. Amongst the many factors involved in coordinating these processes in bone, the insulin-like growth factors appear to play an important role. Not only have IGF-I and IGF-II been shown to directly stimulate a range of markers for cell proliferation and protein synthesis in cartilage and bone cells isolated from foetal bone, but their importance in overall foetal bone growth and development has been clearly demonstrated in mice, where an absence of either factor results in delayed growth plate maturation and reduced foetal bone growth (see Chapter 1). The relationship between IGF-I and IGF-II and their availability in areas of bone and cartilage to achieve these actions during foetal growth and development were the subject of the present study.

The major findings of the work presented in this thesis can be summarised as follows. IGF-II was highly expressed and available in those areas of bone where chondrogenesis and osteogenesis takes place during foetal development. In the growth plate, IGF-II was co-localised with IGF-I and IGF-II binding sites in the hypertrophic zone. This binding represented association with the Type I and Type II IGF receptors in chondrocytes, and either IGF BPs or extracellular matrix components in cartilage matrix. IGF BP-2, -3, -4 and -5 were co-localised with IGF-II, but evidence suggested that IGF BP-2, -4 and -5 were not locally produced in the growth plate. In contrast, significant levels of IGF-I did not appear to be locally produced or derived from the circulation in most areas of bone, suggesting that the

levels of IGF-I that were found to co-localise with IGF-II were low. There was however, one area in bone at the boundary between the growth plate and bone, in which significant levels of IGF-I appeared available to cells as a result of uptake from the circulation and by local production.

IGF-II was available to many cells within bone during foetal development in the present study, with localisation suggesting an important role for IGF-II in chondrogenesis and osteogenesis. The presence of IGF-II mRNA in cells where IGF-II peptide was detected indicates that IGF-II is likely to be locally produced in foetal bone, and may therefore have predominantly local actions.

The IGF-II system *in vivo* appears to be very complex, with the production of multiple IGF-II transcripts and the presence of IGF-II peptide in many areas of bone. Chondrocytes located in the hypertrophic zone are the likely targets of IGF-II effects in foetal sheep growth plates, in support of some of the actions observed for IGF-II on chondrocytes *in vitro*. Not only was there a high level of IGF-II expression in these cells, but both IGF-II peptide and IGF receptors were co-localised in this area. These results are supported by the studies of Hill *et al.* (1992a), Hill and De Sousa (1990a) and Luyten *et al.* (1988), who demonstrated stimulatory effects for IGF-II on protein synthesis in chondrocytes derived from foetal growth plates, and from newborn articular cartilage. However, IGF-II appeared much less potent than a range of other growth factors in these culturing systems, suggesting either that IGF-II has major additional, as yet uncharacterised effects on hypertrophic chondrocytes, or that IGF-II is less potent *in vitro* than it is *in vivo*. This may be due to a requirement for other factors, such as IGF BPs to modulate IGF-II action, which may not be available in cell culture.

IGF BPs are likely to be required for the coordination and regulation of IGF activity, to achieve maximal stimulation. In the present study, IGF BPs were also available in those areas where IGF-II was found, and therefore they have potential to participate in the modulation of IGF-II actions during foetal bone development. IGF BP-2, -3, -4 and -5 were detected in hypertrophic chondrocytes in the growth plate, and although there was no evidence for their

presence in the cartilage matrix using immunohistochemistry, results from the IGF-II radioligand binding assay suggest IGF BPs were also located in cartilage matrix. IGF BP-2 and-5 have preference for IGF-II and have been shown to potentiate IGF actions in some cell systems (Jones and Clemmons, 1995). In particular, Bautista *et al.* (1991) showed that coincubation of mouse osteoblasts with equimolar concentrations of IGF-II and IGF BP-5 resulted in an increase in the degree of IGF-II stimulation in these cells.

In areas where osteogenesis was taking place, IGF-II appeared capable of stimulating both the differentiation of osteoblasts, and the proliferation of pre-osteoblasts. Few studies have characterised actions for IGF-II in specific cells from the bone cell lineage, which is a little surprising given the finding that IGF-II is one of the most abundant growth factors found in bone (Frolik *et al.*, 1988; Mohan *et al.*, 1988a; Mohan *et al.*, 1986). Furthermore, results from this and other studies find a high level of IGF-II expression in osteoblasts at various stages of differentiation (Wang *et al.*, 1995; Shinar *et al.*, 1993), and abundant IGF-II is secreted by cultured osteoblsasts (McCarthy *et al.*, 1992; Mohan *et al.*, 1990; Mohan *et al.*, 1988b). One study has, however, demonstrated that osteoblasts in calvarial cultures are capable of responding to IGF-II, with a modest stimulatory effect on matrix protein production (Mohan and Baylink, 1991a and b; McCarthy *et al.*, 1989a). Further studies are required to determine the significance of IGF-II localisation in particular bone cells found in the present study.

Results from the present study support a role for the extracellular matrix in the storage of IGF-II in bone. IGF-II was located in bone matrix in all areas during foetal development, which may account for the levels of IGF-II able to be extracted from human bone postnatally (Frolik *et al.*, 1988; Mohan *et al.*, 1988a; Mohan *et al.*, 1986). A range of other growth factors including IGF-I have also been found specifically in bone matrix (Slater *et al.*, 1994; Hauschka *et al.*, 1986). It is postulated that IGF storage in the bone matrix is a mechanism by which bone formation and bone degradation are coupled (Margolis *et al.*, 1996; Rosen *et al.*, 1994; Mohan and Baylink, 1991a and b). However, a clear role for IGF-II in linking these processes is lacking. There is currently no evidence to support a role for IGF-II on osteoclast activity. The significance of this reservoir of IGF-II needs further investigation.

In contrast to IGF-II, in this study there did not appear to be a significant level of local IGF-I production, or uptake of IGF-I from the circulation, in most areas of foetal bone. These results are supported by other studies, which have found low levels of IGF-I mRNA in cells from the bone cell lineage, and an absence of IGF-I mRNA in chodrocytes in the growth plate, of foetal and postnatal rodents (Wang *et al.*, 1995; Shinar *et al.*, 1993). Likewise, the ability for IGF-I to penetrate into areas of articular cartilage has been shown to be very restricted, particularly when bound to IGF BPs (Schneiderman *et al.*, 1995a).

Somewhat surprising therefore, was the detection of IGF-I in the current study in most areas of cartilage and bone. Previous reports in postnatal rat and pig have also described a similar distribution pattern for IGF-I, concluding that IGF-I has important and widespread effects on various cell types in bone (Lazowski *et al.*, 1994; Hansson *et al.*, 1988; Simon and Cooke, 1988; Nilsson *et al.*, 1986). However, in view of the current results, it is unlikely that IGF-I levels are high in very many areas, which is supported by the results of Mohan *et al.* (1988a), who quantified IGF-I in whole bone from postnatal human femurs and found levels to be negligible (0.19 μg IGF-I per g bone powder). The concentration of IGF-I was approximately nine times lower than the level of IGF-II in the same extract (Mohan *et al.*, 1988a). It is therefore questionable whether the levels of IGF-I *in vivo* are sufficient to stimulate the effects observed for IGF-I on cartilage and bone cells which have been observed *in vitro*. Schneiderman *et al.* (1995a) found that the concentration of IGF-I in its free and bound forms in normal articular cartilage was too low to account for the stimulatation of proteoglycan synthesis observed *in vitro* (McQuillan *et al.*, 1986; Guenther *et al.*, 1982). Although this conclusion may be true for normal adult cartilage, in animals where the level or composition of proteoglycan in the matrix is altered, such as in osteoarthritis, it has been demonstrated that there is a marked increase in local IGF-I expression and uptake of IGF-I complexes into articular cartilage, which could be sufficient to stimulate the effects observed *in vitro* (Middleton *et al.*, 1996; Schneiderman *et al.*, 1995b; Middleton and Tyler, 1992). This may also explain results obtained in hypophysectomised animals. GH stimulation in these animals increases the level of IGF-I peptide and IGF-I mRNA to levels that are more abundant than

the normal steady-state levels of IGF-I and IGF-I mRNA in intact animals (Alarid *et al.*, 1992; Isgaard *et al.*, 1988a; Isgaard *et al.*, 1988b; Nilsson *et al.*, 1986). Furthermore, *in vivo* studies have shown significant stimulation of bone growth in hypophysectomised rats when IGF-I is introduced directly into the growth plate or via the circulation, in contrast to minimal effects for IGF-I on bone growth in intact animals (Skottner *et al.*, 1987; Isgaard *et al.*, 1986; Schoenle *et al.*, 1985; Russel and Spencer, 1985). These results suggest that in normal animals the level and effectiveness of IGF-I is minimal, however when the composition of the matrix is altered, the availability and therefore the effectiveness of IGF-I to cartilage cells is increased.

The abundance of IGF-I and therefore its potential effectiveness may also be variable depending on the stage of development. Luyten *et al.*, 1988 found that bovine articular cartilage from newborn animals, in contrast to adult humans, contains levels of IGF-I that are able to stimulate proteoglycan synthesis. Further investigations in foetal sheep need to address whether the predicted low level of IGF-I present *in vivo* from the present study, is sufficient to stimulate cells in those areas of bone where it was located, especially given results from targeted gene deletion studies for IGF-I that clearly demonstrate a role for IGF-I in foetal bone growth (Liu *et al.*, 1993; Baker *et al.*, 1993).

It is possible that the area at the boundary between the growth plate and bone could be a major target for the direct actions of IGF-I in foetal sheep bone, which may result in a flow on effect to other cells in bone, resulting in the phenotype observed in the foetal period by Liu *et al.* (1993) and Baker *et al.* (1993) in mice with an IGF-I gene deletion. Compared to other areas, this zone was likely to contain high levels of IGF-I during bone development, since circulating IGF-I and IGF-I expression were found predominantly in this area in the present study. The effects of IGF-I on hypertrophic chondrocytes, endothelial cells, osteoblasts and osteoclasts that are located in this area, have been reported in numerous *in vitro* studies (Goad *et al.*, 1996; Formigli *et al.*, 1995; Panagakos, 1993; Hill *et al.*, 1992a; Ohlsson *et al.*, 1992a; Mochizuki *et al.*, 1992; Slootweg *et al.*, 1992; Streeten and Brandi, 1990; Demarquay *et al.*, 1990; Pfeilschifter *et al.*, 1990; VandePol *et al.*, 1989; Hock *et al.*, 1988; Schmid *et al.*, 1984;

Hill, 1979). It is becoming clear that the invasion of capillaries into the metaphyseal end of the growth plate is a key part of endochondral ossification, and the replacement of cartilage with mineralised bone, during bone growth (Alini *et al.*, 1996; Goad *et al.*, 1996; Formigli *et al.*, 1995). IGF-I has been postulated to provide the necessary link between endothelial cells, osteoclasts and osteoblasts to coordinate this process (Goad *et al.*, 1996; Formigli *et al.*, 1995; Hill *et al.*, 1995).

Overall, results from the present study suggest that the direct effects of IGF-I and IGF-II are likely to be different in foetal sheep bone development, as a result of a difference in their availability to particular cells in cartilage and bone. The finding that the effects of IGF-I and IGF-II in chondrocyte and osteoblast cell cultures are similar, is likely to be due to their common action through the Type I IGF receptor (LeRoith *et al.*, 1995; Jones and Clemmons, 1995; Lau *et al.*, 1994; Liu *et al.*, 1993). Therefore results obtained *in vitro* do not necessarily reflect the availability of IGF-I and IGF-II to these cells *in vivo*.

7.1 FUTURE DIRECTIONS

It will be essential to determine if the level of IGF-I in foetal bone at particular locations, such as at the boundary between the growth plate and bone, is sufficient to enable cells where IGF-I is located to respond. The relative level of circulating IGF-I entering the zone at the boundary between the growth plate and bone could be analysed in foetal sheep *in utero*, by lengthy infusion of N-met IGF-I to reach a constant steady-state in foetal circulation. The level of N-met IGF-I accumulating and therefore available in bone could be analysed following infusion.

IGF-II action in foetal bone has been poorly defined, but results from the present study suggest clearly that it is available to many areas in bone. The heterogeneity in IGF-II expression in bone from various promoters needs further investigation. Defining the function and significance of alternate promoter usage and identifying which promoters are active in bone will assist in determining factors that could regulate local IGF-II action. Furthermore, the contribution of IGF-II from the ciruclation would reveal whether this source of IGF-II is

available to bone and if circulating IGF-I and IGF-II have potential actions at the same location.

APPENDIX

GENERAL MOLECULAR BIOLOGY TECHNIQUES

In addition to the main methodology described in Chapter 2, general techniques that are associated with manipulating nucleic acids and protein, subcloning cDNA and maintaining plasmid stocks, and histological staining were used. These methods are described below since although important they are peripheral to the main methods required to achieve the results reported in this thesis. The source of materials and the content of specific solutions not mentioned here are described in Chapter 2. Unless otherwise mentioned, the methodology described is sourced from a standard molecular biology laboratory manual (Sambrook *et al.*, 1989; Maniatis *et al.*, 1982) or a standard histology text (such as Lillie, 1965).

RESTRICTION ENZYME DIGESTS

Each restriction endonuclease reaction contained the following components (added in order): appropriate 10 X restriction enzyme buffer (supplied with restriction enzyme) to 10% of the final reaction volume, DNA (the amount determining the final reaction volume and enzyme concentration), Milli Q water to the appropriate final reaction volume, and a suitable enzyme(s) at less than 10% w/v of the final reaction volume. The mixture was incubated at 37°C (or other temperatures where necessary) for an appropriate length of time given the DNA and enzyme concentrations. Restriction was stopped by denaturing the enzyme for 5 minutes at 65°C. Digestion of the DNA was checked by non-denaturing gel electrophoresis and the DNA purified by ethanol precipitation.

GEL ELECTROPHORESIS

Nucleic Acids

Non-Denaturing Gel Electrophoresis

DNA was analysed by electrophoresis to determine the size of different fragments and to allow isolation and purification of specific DNA species. Agarose gel electrophoresis of DNA was carried out in submerged gels for 1 - 3 hours at 50 - 100 volts. DNA was visualised on an ultraviolet transilluminator screen and photographed.

Agarose Gel High melting point agarose (generally 1% w/v) was added to agarose gel running buffer (TAE buffer) and dissolved at temperature. After cooling to 65°C the mixture

was poured into a gel former. Ethidium bromide was added to a final concentration of 0.5 µg/ml.

Agarose Gel Loading Buffer Tracking dyes (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol) 15% w/v Ficoll and Milli-Q water to produce a 10 X stock concentration.

Agarose Gel Running Buffer (TAE) (Maniatis *et al.*, 1982) 40 mM Tris-acetate; 1 mM EDTA [pH 8.0] pH approximately 7.6 with glacial acetic acid. Ethidium bromide was added to a final concentration of 0.5 µg/ml.

Denaturing Gel Electrophoresis

Denaturation of RNA secondary structure is required to enable good size separation and resolution by gel electrophoresis (Davis *et al.*, 1986). Gel electrophoresis of RNA was carried out in agarose/formaldehyde gels.

Formaldehyde Agarose Gel 1 % - 1.5% high melting point agarose, 2.2 M formaldehyde, 20 mM (N-morpholine)-propane-sulphonic acid (mops), 5 mM sodium acetate and 0.5 M EDTA (pH 7.0). The agarose in water was melted and sterilised by autoclaving and cooled to 65°C before the other components were added.

Formaldehyde Gel Loading Buffer (Lehrach *et al.*, 1977) 50% formamide, 20 mM mops, 5 mM sodium acetate, 0.5 mM EDTA, 2.2 M formaldehyde, tracking dyes (0.001% w/v Bromophenol blue, 0.001% w/v Xylene cyanol).

Formaldehyde Gel Running Buffer (Davis *et al.*, 1986) 20 mM MOPS; 5 mM sodium acetate; 0.5 mM EDTA, pH between 5.5 and 7.0. 2.2 M formaldehyde was added after autoclaving.

Prior to loading, samples were vortexed, boiled for 1 minute and chilled on ice. Electrophoresis was carried out for 5 - 8 hours at 50 - 100 volts.

Proteins

SDS Polyacrylamide Gel Electrophoresis

Isolated protein or affinity cross-linked products were electrophoretically separated on an SDS polyacrylamide gel according to the method of Laemmli (1970) and described fully by Deutsher (1990).

Separating Gel

(7.5%) 0.375 M Tris, pH 8.8; 0.1% w/v SDS; 25% v/v stock acrylamide/bis; 0.05% w/v ammonium persulphate; 0.001% v/v N,N,N,N-tetramethylethylenediamine (TEMED)

(15%) 0.375 M Tris, pH 8.8; 0.1% w/v SDS; 50% v/v stock acrylamide/bis; 0.05% w/v ammonium persulphate; 0.001% v/v TEMED

Stacking Gel

(4%) 0.125 M Tris, pH 6.8; 0.1% w/v SDS; 13.4% v/v stock acrylamide/bis; 0.05% w/v ammonium persulphate; 0.001% v/v TEMED

(Stock Acrylamide/Bis: 29.2% w/v acrylamide; 0.8% w/v bis/acrylamide)

Protein Loading Buffer (2X)

2% w/v SDS; 62.5 mM Tris, pH 6.8; 0.1% v/v β-mercaptoethanol; 0.001% w/v bromophenol blue; 0.1% glycerol

Protein Running Buffer

0.025 M Tris (pH 8.3); 0.192 M glycine; 0.1% w/v SDS

Protein Transfer Buffer

25 mM Tris; 0.2 M glycine; 20% methanol

Samples were prepared as described in the appropriate section and loaded into the wells in the stacking gel using a fine syringe. Not more than 50 µl per well was loaded and the syringe was washed thoroughly between each well loading.

Following electrophoresis, gels were soaked in protein transfer buffer for 20 minutes. Protein transfer from SDS polyacrylamide gels to nitrocellulose membranes was carried out using a semi-dry horizontal electrophoretic transfer method. On top of the saturated graphite plate of the anode electrode was placed chromatography paper, the SDS polyacrylamide gel, nitrocellulose membrane and chromatography paper all pre-wet in protein transfer buffer. The unit was assembled and a current of 8 mA/cm was applied for approximately 1 hour.

PURIFICATION OF RNA OR DNA

Extraction with Phenol-Chloroform

To each sample (DNA), an equal volume of phenol was added. After mixing by vortex and centrifugation for 1 minute at 13 000 rpm, the upper aqueous phase was transferred to a fresh tube. An equal volume of a 1:1 mixture of phenol:chloroform was added to the separated aqueous phase. After thorough mixing and centrifugation for 1 minute at 13 000 rpm, the upper aqueous phase was placed in a fresh tube. An equal volume of chloroform was added. After mixing by vortex and centrifugation for 1 minute at 13 000 rpm, the aqueous phase was transferred to a fresh tube. This process was repeated until protein was no longer visible at the interphase. RNA samples were treated similarly except phenol:chloroform:isoamyl alcohol (25:24:1) was substituted for phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1) was substituted for chloroform.

Ethanol Precipitation

Ethanol precipitation is a standard method for concentrating DNA or RNA. To each DNA or RNA sample, an appropriate volume of either sodium acetate (0.25 M final concentration), sodium chloride (0.1 M final concentration) or ammonium acetate (2.0 M final concentration) was added. Ethanol at 2 volumes for DNA or 2.5 volumes for RNA was added and mixed. If the DNA or RNA concentration was low, tRNA carrier (10 µg/µl) was added to increase recovery, by facilitating precipitation (Davis *et al.*, 1986). After a minimum of 10 minutes at -20°C, the sample was centrifuged at 13 000 rpm for at least 10 minutes. The supernatant was discarded and the pellet and tube washed at least once with ethanol at 70%. After drying the pellet under vacuum, the DNA or RNA was resuspended in the desired volume of buffer and heated for 5 minutes at 65°C to aid resuspension.

Purification of DNA from Agarose Gels (Braesatec DNA purification kit)

The appropriate band was excised from the agarose gel. To this was added 3 volumes (1 volume = 1 g agarose) of BRESA-SALT™ and the agarose melted at 55°C for 5 minutes. Once melted, 5 µl plus 1 µl/µg DNA of BRESA-BIND™ was added and the mixture incubated for 5 minutes at room temperature. The mixture was spun for 5 seconds at 13 000 rpm and the supernatant removed. The pellet was washed in BRESA-WASH™ at a volume equivalent to the initial volume of DNA and BRESA-SALT™. The mixture was again spun for 5 seconds at 13 000 rpm and the supernatant removed. The DNA was recovered by resuspending the pellet two times in TE buffer (pH 7.6) and pooling the supernatants.

SPECTROPHOTOMETRY

DNA or RNA concentration was measured at λ 260 nm. Purity of RNA was determined as the ratio of absorbance at λ 260 and 280 nm. High RNA purity exists if the ratio is approximately 2.0. Bacterial cell density was measured λ 600 nm. Stationary phase was represented by an absorbance of 1.0 or above.

INCORPORATION OF RADIOLABEL INTO RNA OR DNA

Incorporation of radiolabel into RNA or DNA was measured as follows: 0.5 µl sample was placed in 20 µl 0.5 M EDTA, pH 8.0. Onto two pieces of Whatman 1 MM filter paper 10 µl was spotted. One piece was washed in ice-cold 5% trichloroacetic acid solution, for 2 periods of 5 minutes each. Both pieces were dried and then placed in 400 µl scintillation fluor. Activity on each filter paper was measured in a liquid scintillation counter. The ratio of

activity measured for washed (radioactivity incorporated into nucleic acids) and unwashed (total radioactivity) was the measure of percentage incorporation of radiolabel into transcripts. Typical incorporations ranged from 40% to 95%.

TRANSFORMATION OF *Escherichia coli* WITH PLASMID DNA

Production of Competent Cells (Inoue *et al.*, 1990)

Frozen *Escherichia coli* (strain DH5α) was thawed and streaked onto an LB plate and cultured overnight at 37°C. Ten to twelve colonies (2-3 mm in diameter) were isolated and inoculated into 250 ml of LB broth. The culture was grown to an absorbance of 0.6 (λ 600) at 18°C, with vigorous shaking. After the appropriate cell density was reached, the culture was placed on ice for 10 minutes following which the cells were pelleted at 2 500 X g for 10 minutes at 4°C. The pellet was resuspended in 80 ml of ice-cold transformation buffer (10 mM HEPES; 15 mM $CaCl_2$; 250 mM KCl; KOH was added until pH 6.7 was reached, following which 55 mM $MnCl_2$ was added), and incubated on ice for 10 minutes prior to re-centrifugation at 2 500 X g for 10 minutes. The cell pellet was gently resuspended in 20 ml of transformation buffer and DMSO was added to a final concentration of 7%. After incubating for a further 10 minutes on ice, the competent cells were ready for use. Aliquots were frozen in liquid nitrogen and stored at -80°C until required.

Subcloning DNA Fragments with Cohesive or Blunt Ends

Plasmid DNA and the cDNA sequence were cut with the appropriate restriction enzymes to yield compatible ends. The DNA bands to be ligated were then purified from agarose after gel electrophoresis was carried out to separate the restricted fragments. To 10 ng of cut plasmid DNA, between 1 and 4 ng of cut cDNA, 10% v/v ligase buffer (supplied with the enzyme) and 1 U T4 DNA ligase were added. A series of control reactions excluding plasmid DNA, and/or cDNA or T4 DNA ligase were also performed. Ligation was carried out overnight at 15°C following which bacteria were transformed and grown to amplify the product. Ligated products were then selected on LB plates containing the appropriate antibiotic.

Transformation of *E. coli*

To 100 µl of ice-cold competent cells, 10 ng of DNA (ligated product to be transformed) was added and incubation carried out on ice for 30 minutes. The cells were heat-shocked by placing at 42°C for 5 minutes. After cooling on ice for 2 minutes, 900 µl LB broth was added. Cells were then incubated at 37°C for 1 hour with shaking. Finally the cells were plated onto agar plates supplemented with the appropriate antibiotic and grown overnight at 37°C.

Plasmid DNA from selected colonies was isolated by the small-scale isolation procedure described below and the integrity of plasmid DNA was characterised by restriction enzyme digestion and gel electrophoresis.

SMALL-SCALE ISOLATION OF PLASMID DNA

Into 1.2 ml of Luria-Bertani medium containing the antibiotic ampicillin (10 mg/ml) was inoculated the appropriate colony grown on an agar plate. The culture was grown overnight at 37°C in a shaking incubator. The cells were pelleted by spinning at 13 000 rpm for 1 minute. After removing the supernatant, 200 µl of a solution containing (8% w/v sucrose; 0.5% w/v triton X-100 50 mM EDTA [H 8.0] 10 mM Tris [H 8.0]) was added. To this, 25 µl of fresh lysozyme (10 mg/ml) was added. After mixing, the sample was boiled for 40 seconds and then spun at 14 000 rpm for 10 minutes. The pellet was removed with a toothpick. To the supernatant, 40 µl of 2.5 M sodium acetate and 420 µl of isopropanol were added and the DNA precipitated in the usual way. Characterisation of plasmid DNA was by agarose gel electrophoresis.

LARGE-SCALE ISOLATION OF PLASMID DNA

Recombinant plasmid DNA containing templates for probe generation were isolated from *Escherichia coli* by the following procedure of Birnboim (1983).

Into 50 ml of Luria-Bertani medium containing the antibiotic ampicillin (500 µl, 10 mg/ml), 500 µl of stock (*Escherichia coli* HB101 strain containing plasmid with the appropriate cDNA insert) was aseptically added. The culture was grown to stationary phase overnight at 42°C in a shaking incubator. Stationary phase was determined by spectrophotometry at λ 600 nm. The culture was centrifuged at 4 000 rpm for 10 minutes at 4°C. After removing the supernatant, the cells were resuspended with shaking in 1.5 ml of ice-cold lysozyme buffer. After adding lysozyme (59 U/µl), the mixture was held on ice for 30 minutes. A 0.5 ml volume of a solution containing 0.2 N hydrochloric acid and 0.1% sodium dodecyl sulphate was added and mixed by continuous inversion of the tube. The mixture was stored at room temperature for 5 minutes and on ice for a further 20 minutes, after which 1.9 ml of ice-cold potassium acetate (300 mM, pH 4.8 with glacial acetic acid) was added. After mixing, the preparation was stored on ice until a flocculent white precipitate formed. The bacterial lysate was centrifuged at 12 000 rpm for 15 minutes at room temperature. The supernatant was transferred to a fresh tube and 3.6 µl propan-2-ol added. After mixing, the tube was left at

room temperature for 5 minutes. Centrifugation was then carried out at 10 000 rpm for 10 minutes. After discarding the supernatant, the pellet and tube were rinsed twice with ethanol at 70°C and resuspended in 150 µl TE buffer (pH 7.5) and 250 µl lithium chloride (5 M). The mixture was stored at 4°C for a minimum of 30 minutes, and then centrifuged at 13 000 rpm for 5 minutes. The supernatant was transferred to a fresh tube and ethanol precipitation was carried out using sodium acetate. The pellet was resuspended in 100 µl TE buffer (pH 8.0). Concentration of the plasmid DNA was determined spectrophotomically at λ 260 nm and 280 nm. Characterisation of plasmid DNA was by agarose gel electrophoresis.

Luria-Bertani Broth (LB broth) (Davis *et al.*, 1986)

Bacto-tryptone (10 g), Bacto-yeast extract (5 g), NaCl (10 g) and Milli Q water to 1 L were mixed together and the pH adjusted to 7.5 with NaOH. For agar plates, 1.5% agar was added in the medium prior to autoclaving. The appropriate antibiotic (usually ampicillin) was aseptically applied (150 µl; 10 mg/ml) to the agar surface of each agar plate.

Lysozyme Buffer (Birnboim, 1983)

50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)

DETERMINATION OF Tm AND HYBRIDISATION TEMPERATURE

Factors that affect the stability of cRNA-mRNA hybrids and hence the specificity of the reaction need to be optimised. These factors can be represented by an equation developed for DNA-DNA hybridisation in solution or on filter (Thomas and Dancis, 1973). This equation was adapted for RNA-RNA hybrids (Angerer *et al.*, 1987). It has been shown to mostly hold true for *in situ* hybridisation with a slight depression of 5°C from the calculated Tm of hybrids formed *in situ* (Brahic and Haase, 1978; Cox *et al.*, 1984; Kelsey *et al.*, 1986).

$$Tm = 79.8 + 58.4\ (F_{GC}) + 11.8\ (F_{GC})^2 + 18.5 \log (M) - (820 \div L) - 0.35\ (\%\ F) - (\%\ m)$$

Tm = temperature at which half of the hybrid molecules separate into single strands

F_{GC} = mole fraction guanosine-cytosine content (approximately 40 to 50%)

M = monovalent cation concentration (1.08M)

L = length of duplexes formed (number of base pairs)

% F = percentage of formamide v/v

% m = percentage of non-complementary base pairs in the duplex formed (detection of homologous sequences, % m = 0)

The variable components in the equation have the following effect on RNA-RNA hybrid stability. Increasing ionic strength has a stabilising effect on RNA-RNA hybrids by decreasing solubility of bases and hence increasing hydrophobic interactions. Also, an increase in ions neutralises the negative charge on nucleic acids thus removing the repulsive force between RNA strands approaching to form hybrids (Coghlan *et al.*, 1987). A higher percentage of guanosine-cytosine pairs increases stability of the hybrid since each guanosine-cytosine pair contributes three hydrogen bonds as opposed to two for adenosine-uracil pairs (Coghlan *et al.*, 1987). Formamide destabilises RNA-RNA hybrids by disrupting hydrogen bonds (Jones and Robertson, 1970; Casey and Davidson, 1977). Increased probe length theoretically stabilises RNA-RNA hybrids by an increase in the number of hydrogen bonds. However, in choosing optimum probe length, base mismatching between strands needs to be considered (Bonner *et al.*, 1973) along with decreased accessibility to target RNA with long probes.

In general, it is recommended that *in situ* hybridisation be carried out under low stringency conditions at an optimum temperature of Tm minus 25°C (Angerer *et al.*, 1987). In the present study, the appropriate hybridisation temperature was determined empirically for each probe based on the theoretical Tm.

HISTOLOGICAL PROCEDURES

Section Adherence

All slides were cleaned in chromic acid (50% w/v sodium dichromate; 50% sulphuric acid) and rinsed well prior to coating with adhesive.

Aminoalkylated glass slides

Slides were immersed in fresh 2% 3-aminopropyltriethoxysilane (diluted in acetone made dry with $MgSO_4$) for 5 seconds followed by two washes in dry acetone. After two further washes in milli Q water, slides were dried in an oven.

Gelatine-Chrome Alum

Slides were dipped in 0.5% w/v gelatine (containing 0.05% w/v chromium potassium sulphate) up to three times. Slides were dried in an oven between each coating.

Staining Procedures

alcian blue

Sections from the receptor binding assay were first stained in alcian blue for 20 minutes. They were then washed in running tap water (30 seconds) before being counterstained with haematoxylin and eosin.

haematoxylin (Gills or Erlichs) and eosin

Exact staining times were determined empirically. Slides were passed through the following procedure: haematoxylin (1 - 30 minutes); acid/alcohol (if using Erlichs haematoxylin) (2 minutes); 'blue' in running tap water (with an optional addition of 1% ammonia) (5 - 20 minutes); eosin (1 - 2 minutes); tap water (30 seconds); dehydration through an alcohol series of increasing ethanol concentrations (70%, 95%, 96%, 96%, 30 seconds each); 50% xylene (or toluene)/50% ethanol (30 seconds); xylene (or toluene) (30 seconds); xylene (or toluene) (30 seconds). After checking the intensity of staining with a microscope, sections were mounted in D.P.X.

haematoxylin (Carazzis)

Slides were passed through the following procedure: haematoxylin (2 minutes); 'blue' in running tap water (5 minutes); dehydration through an alcohol series of increasing ethanol concentrations (70%, 95%, 96%, 96%, 30 seconds each); 50% xylene (or toluene)/50% ethanol (30 seconds); xylene (or toluene) (30 seconds); xylene (or toluene) (30 seconds. After checking the intensity of staining with a microscope, sections were mounted in Eukitt.

Stock Stains

acid/alcohol

0.2% v/v HCl; 70% ethanol (1:4)

alcian blue

0.1% w/v alcian blue; 3% v/v acetic acid

Carazzi's haematoxylin

0.25 g haematoxylin, 50 ml glycerol, 12.5 g aluminium potassium sulphate, 0.05 g potassium iodate water to 250 ml

eosin (Humason, 1979)

To 95 ml of ethanol at 70%, 1 g of eosin Y and 5 ml of glacial acetic acid were added.

Gill's haematoxylin (Gill, Frost and Miller, 1974) A 1 L solution of haematoxylin was made. Haematoxylin, anhydrous (2.0 g); sodium iodate (oxidant, 0.2 g); aluminium sulphate (mordant, 17.6 g); distilled water (730 ml); ethylene glycol (250 ml); glacial acetic acid (20 ml).

Erlichs haematoxylin

2% v/v haematoxylin in methanol mixed 1:1 with 12.4% aluminium ammonium sulphate. Mix 2:1 with glycerin, then 4% sodium iodate in glacial acetic acid (30:1 acid solution). Prior to use, haematoxylin was diluted 1:3 with distilled water.

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