INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs) IN GROWTH AND DEVELOPMENT OF THE OVINE FETUS

by

Jillian M. Carr, B.Sc (Hons)

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Department of Biochemistry

University of Adelaide

South Australia

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SUMMARY

The insulin-like growth factor binding proteins (IGFBPs) are important modulators of the IGFs, whose actions are essential for normal fetal growth and development. The extent to which the actions of IGFs are influenced by IGFBPs, in the fetus, are unclear. This thesis has described the ontogeny of IGFBPs in the sheep, *in utero*, throughout normal development and following placental restriction of substrate delivery to the fetus, and has furthered our understanding of the roles of IGFBPs in the fetus. Circulating IGFBP-3, the major IGFBP post-natally and IGFBP-2, which preferentially binds IGF-II (the more abundant IGF *in utero)*, were quantitated by Western ligand blotting. Additionally, IGFBP-4 was investigated, since at the time of commencement of this project it was a novel IGFBP that had been purified and characterised by collaborators and was readily detectable by Western ligand blotting. IGFBP-3 was also measured by radioimmunoassay (RIA). Tissue mRNA levels of IGFBP-2 and -4 were quantitated by Northern analysis and plasma IGF-I and -II were measured by RIA.

In order to detect and quantitate oIGFBP-4 mRNA levels, oIGFBP-4 cDNA clones were isolated. Partial oIGFBP-4 cDNA clones were generated by library screening and N-terminal sequences identified by reverse transcription polymerase chain reaction (RT-PCR). The DNA and protein sequences for oIGFBP-4 has provided novel sequence data in this species, which shows strong identity with sequences published for IGFBP-4 from human, rat and cow (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a; Moser *et al.*, 1992). An antibody to oIGFBP-4 was generated by immunising rabbits with peptides directed against specific regions of oIGFBP-4. The antigenicity of these peptides were enhanced through the use of a hybrid bacteriophage display system (Greenwood *et al.*, 1991). The antibody produced was specific for oIGFBP-4 without cross reactivity with other IGFBPs and may be useful for the future development of an oIGFBP-4 RIA or other techniques to further characterise this protein.

Changes in IGFBP abundance were demonstrated throughout development and with restricted fetal substrate supply and growth. Circulating IGFBP-2 rose in early to mid

gestation, then declined between late gestation and 1 day post-natally. These changes paralleled those in liver and kidney IGFBP-2 mRNA and circulating IGF-II. In the restricted fetus, the ontogeny of circulating IGFBP-2 was altered, with prematurely elevated levels in mid gestation and an early decline in late gestation. These changes in circulating IGFBP-2 were negatively correlated with fetal body and organ weights, suggesting an inhibitory action of circulating IGFBP-2 on fetal growth. Changes in circulating IGFBP-2 in the growth restricted fetus again paralleled reported levels for circulating IGF-II (Jones *et al.*, 1988; Owens *et al.*, 1994), as was observed in the normal fetus. These correlations suggest the circulating ontogenic changes in IGFBP-2 may be mediated by or co-regulated with IGF-II, possibly by factors shown to regulate IGF-II *in utero*, such as cortisol (Li *et al.*, 1993). Circulating fetal IGFBP-3 and IGFBP-4 rose as gestation progressed and were correlated with circulating IGF-I, and for IGFBP-4 with circulating IGF-I and liver IGFBP-4 mRNA levels. These findings suggest that IGF-I may regulate or be co-regulated with IGFBP-3 and -4, *in utero*. Circulating IGFBP-4 was elevated in restricted fetuses in mid-late gestation in comparison to normal fetuses, while IGFBP-3 levels were unchanged. Circulating IGFBP-3 and IGFBP-4 were positively associated with fetal body or organ weights, suggesting that these IGFBPs may be associated with stimulation of fetal growth. The changes in circulating IGFBP-3 and IGFBP-4 contrast with a reported decline in IGF-I levels (Owens *et al.*, 1994) suggesting that IGFBP-3 and -4 are under different influences than IGF-I during restriction *in utero*. The observed changes in abundance of IGFBPs may in part be responsible for the phenotype of intra-uterine growth retardation (IUGR), through a combination of decreased levels of IGFs, which act as growth promoters, and increased levels of some IGFBPs, particularly IGFBP-2, which may act as a growth inhibitor.

To further define the role of IGFBPs in growth and development, a comparative study of IGFBPs was undertaken in the marsupial. In this species, growth and development primarily occurs in the pouch during lactation, which contrasts to eutherian mammals, such as the sheep, that develop in a protected environment and are influenced by the placenta. Characterisation of circulating IGFBPs and IGFBP tissue mRNAs show that marsupial IGFBPs are very similar to those in eutherian mammals. Circulating IGFBPs in the pouch

young undergo a developmental increase co-incident with an acceleration of growth rate and altered nutritional intake through a switch from suckling to grazing.

In conclusion, this thesis describes the isolation and characterisation of oIGFBP-4 cDNA sequence and the generation of a specific oIGFBP-4 antibody. These provide us with useful tools for further characterisation of oIGFBP-4 at the level of the protein and gene. The ontogeny of IGFBPs in normal and growth restricted fetuses has also been characterised. Results indicate a strong association of IGF-II with IGFBP-2 and suggest that the IGFBPs may contribute to fetal growth restriction. Finally, I have characterised IGFBPs in a marsupial which may prove useful as a comparative model for investigation of the endocrine and environmental stimuli that regulate IGF and IGFBPs throughout growth and development.

STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief it contains no material that has previously been published by any other person except where due reference is made. The author consents to the thesis being made available for photocopying and loan.

Jillian Carr

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ABBREVIATIONS

The following abbreviations were used in addition to those abbreviations commonly accepted.

3-[cyclohexylamino]-1-propanesulfonic acid	CAPS
5-bromo-4-chloro-3-indolyl phosphate	BCIP
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	BCIG
acetate	OAc
acid labile subunit	ALS
activator protein	AP : AP-1 and AP-2
analysis of variance	ANOVA
avian myeloid leukaemia virus	AMV
bovine serum albumin	BSA
calf intestinal alkaline phosphatase	CIP
co-efficient of variation	CV
Co-operative research centre	CRC
dimethyl formamide	DMF
dimethyl sulphoxide	DMSO
disuccinyl suberate	DSS
dithioerythritol	DTE
dithiothreitol	DTT
epidermal growth factor	EGF
ethylenediaminetetraacetic acid	EDTA
fibroblast growth factor	FGF
growth hormone	GH
hepatic nuclear factor-1	HNF-1
high efficiency transfer system	HET-S
insulin-like growth factor	IGF : IGF-I and IGF-II
insulin-like growth factor binding protein	IGFBP : IGFBP-1 to IGFBP-6

intra-uterine growth retardation	IUGR
isopropyl-β-D-thiogalactopyranoside	IPTG
least squares means	lsmeans
Luria-Bertani	LB
multiplication stimulating activity	MSA
murine myeloid leukaemia virus	MMV
nitro blue tetrazolium	NBT
non-suppressible insulin-like activity	NSILA
normal adult ewe plasma	NEP
ovine IGFBP-4	oIGFBP-4
parathyroid hormone	PTH
phage storage buffer	PSB
phosphate-buffered saline	PBS
phospho-enol pyruvate carboxy kinase	PEPCK
plaque forming units	pfu
platelet derived growth factor	PDGF
polyethylene glycol	PEG
polymerase chain reaction	PCR
polynucleotide kinase	PNK
polyoxyethylene-sorbitan monolaurate	Tween-20
polyvinylidene difluoride	PVDF
polyvinylpyrrolidone	PVP
radioimmunoassay	RIA
rapid amplification of cDNA ends-PCR	RACE-PCR
reverse transcription-PCR	RT-PCR
SDS-polyacrylamide gel electrophoresis	SDS-PAGE
sodium dodecyl sulphate	SDS
thyroid stimulating hormone	TSH
transforming growth factor-β	TGF-β

trichloroacetic acid	TCA
triiodothyronine	T_3
Tris-(hydroxymethyl)methylamine	Tris
two dimensional	2-D
ultra violet	UV

PUBLICATIONS ARISING FROM THIS THESIS

Carr, J. M., Grant, P. A., Francis, G. L., Owens, J. A., Wallace, J. C. and Walton, P. E. (1994) Isolation and characterisation of ovine IGFBP-4 : protein purification and cDNA sequence. *J. Mol Endocrinol.* **13 :** 219 - 236

Carr, J. M., Owens, J. A., Grant, P. A., Walton, P. E., Owens, P. C. and Wallace, J. C., (1994) Circulating insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and tissue mRNA levels of IGFBP-2 and IGFBP-4 in the ovine fetus. *J. Endocrinol.* (accepted).

Carr, J. M., Owens, J. A., Baudinette, R. V. and Wallace, J. C. (1994) Characterisation of insulin-like growth factor binding proteins (IGFBPs) in the tammar wallaby, *Macropus eugenii*. *Gen. Comp. Endocrinol.* (submitted)

CHAPTER 1. INTRODUCTION

Investigations of the insulin-like growth factors (IGFs) and the IGF binding proteins (IGFBPs) constitute a rapidly expanding field. This literature review will focus on the IGFs and IGFBPs, their protein and gene structure, function and regulation. Finally, I will summarise the recent literature regarding the roles of IGF and IGFBP in fetal growth and development, which addresses the central issues of this thesis.

1.1 THE INSULIN-LIKE GROWTH FACTORS

1.1.1 HISTORICAL IDENTIFICATION OF IGFs

The IGFs were initially identified based on three independent lines of research. In 1957, Salmon and Daughaday reported a growth hormone (GH) inducible 'sulfation factor', identified as a result of the different abilities of sera from normal and hypophysectomised rats to stimulate ^{35}S-sulfate incorporation into rat cartilage *in vitro*. This 'sulfation factor' was later termed somatomedin (Daughaday *et al.*, 1972; Daughaday *et al.*, 1992). The IGFs were also purified on the basis of an 'insulin-like' bioactivity in serum, that was not suppressible with insulin antibodies (non-suppressible insulin-like activity; NSILA) (Froesch *et al.*, 1963) and through the mitogenic properties of calf serum and BRL-3A conditioned medium (multiplication stimulating activity; MSA) (Pierson and Temin, 1972). The subsequent purification and analysis of somatomedin C (van Wyk *et al.*, 1980), MSA (Nissley *et al.*, 1976; Moses *et al.*, 1980) and NSILA (Rinderknecht and Humbel, 1976) confirmed the homology of these three factors, which were later termed IGF based on the structural and sequence similarity to insulin (Figures 1.1 and 1.2).

1.1.2 IGF PROTEIN AND GENE STRUCTURE

The bioactivities described variously as NSILA, MSA or somatomedin C were found to be attributable to one of two closely related peptides : IGF-I or IGF-II. IGF-I and IGF-II are 70 and 67 amino acid peptides, respectively. The sequences of IGF-I and IGF-II

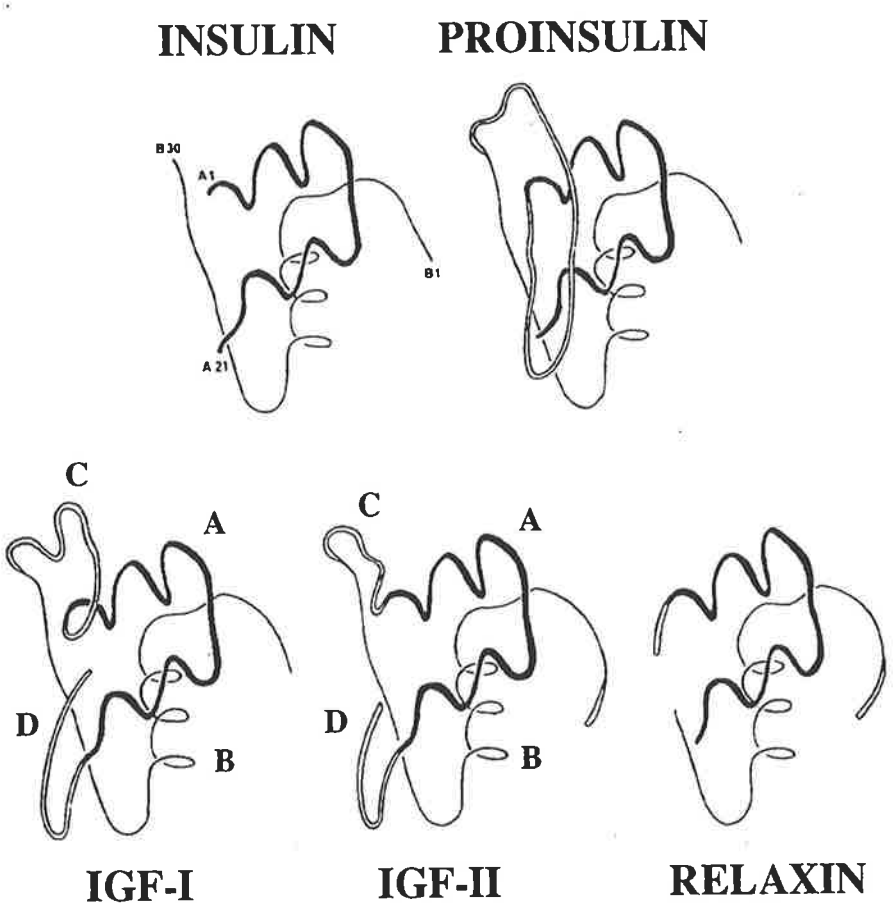


FIGURE 1.1 SCHEMATIC STRUCTURE OF THE INSULIN FAMILY. Predicted folding structures for IGF-I, IGF-II, insulin, proinsulin and relaxin are shown, indicating the structural similarity of the insulin family. The *A*-domain is indicated in black, the *B*-domain by a single line, the *C*-region by the double line between the *A*- and *B*-domains and the *D*-region by the double line extending from the *A*-domain at the C-terminus. Insulin lacks the *C*- and *D*-regions. (Adapted from Blundell and Humbel, 1980)

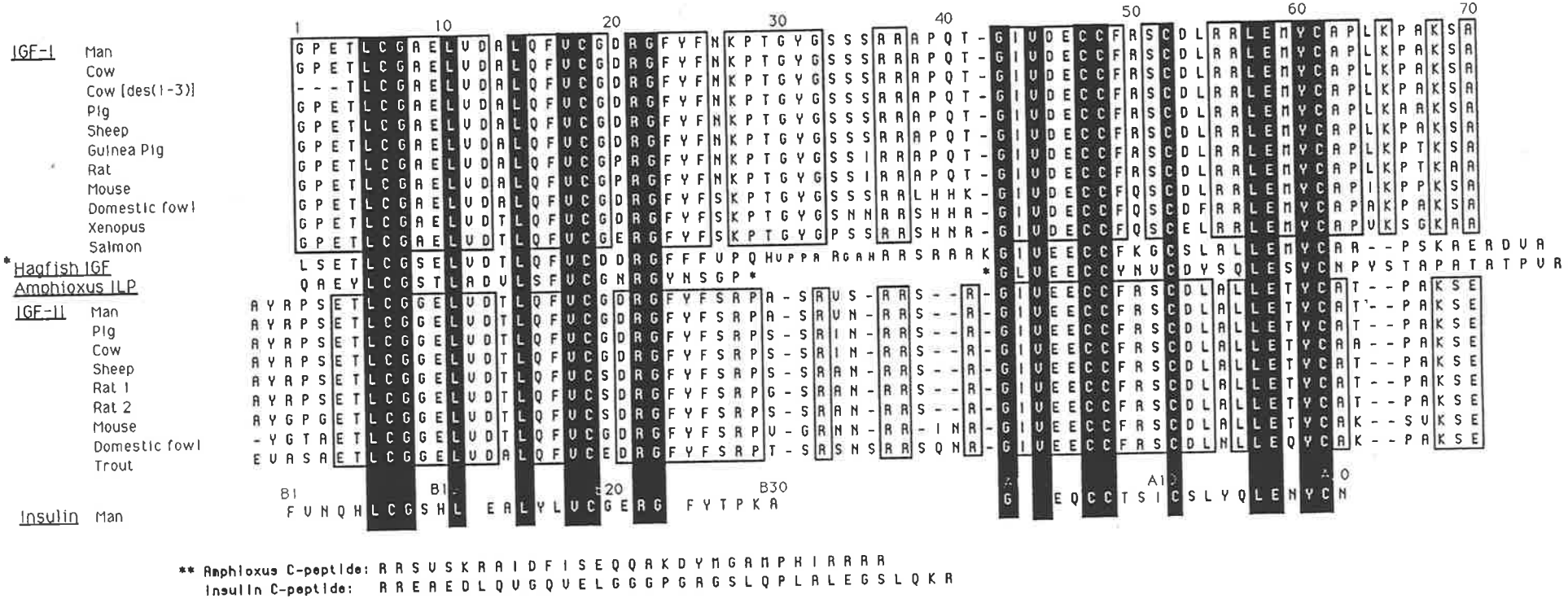


FIGURE 1.2. COMPARISON OF SEQUENCES OF THE INSULIN FAMILY. Protein sequences for IGF-I and IGF-II from several species are aligned and compared with the protein sequence of human insulin. Residues conserved throughout IGF-I, IGF-II and insulin are highlighted with black boxes. Residues conserved between IGF-I and IGF-II are boxed. The alignment shown was generated by a number of co-workers in our laboratory.

peptides are highly conserved across species (Figure 1.2). For example, human and sheep IGF-I and IGF-II differ by only one and four amino acids respectively (Francis *et al.*, 1989). IGF-I and IGF-II also show strong structural homology to each other and with insulin, proinsulin and relaxin, which constitute the insulin family (Blundell and Humbel, 1980) (Figure 1.1). The high degree of sequence conservation between IGF-I, IGF-II and insulin is indicated in Figure 1.2. Of note, are the six cysteine residues which form three conserved disulphide bonds. The structure of the IGF peptides can be described in domains, analogous to the *A, B* and *C* chains of proinsulin. In the mature IGF peptide there is an N-terminal *B*-domain, a *C*-region (homologous to the *C*-peptide cleaved from proinsulin to form insulin), the *A*-domain and a C-terminal *D*-region (Figure 1.1). Additionally, both IGF-I and IGF-II are produced as pre-pro-peptides with a 45 amino acid signal sequence and variable, C-terminal, *E*-peptide extensions (Daughaday and Rotwein, 1989). The different *E*-peptide regions arise from alternate splicing of IGF nuclear RNA to produce multiple mRNA transcripts. Alternative splicing also produces transcripts encoding different N-terminal pre-pro-IGF sequences (Rotwein, 1991; Gilmour, 1994). This complexity is further increased at the level of the gene. The IGF genes are large, with several exons and promoters. Multiple mRNA species for IGF-I and IGF-II arise from alternate promoter usage, which can be regulated by tissue specific, hormonal and developmental factors (Rotwein, 1991; Gilmour, 1994). The human IGF-I gene is estimated to be approximately 90 kb, with six exons and at least two promoters (Jansen *et al.*, 1992). The human IGF-II gene is equally complex, spanning 30 kb of chromosomal DNA with nine exons and at least four promoters (van Dijk *et al.*,1991). The IGF-I and IGF-II genes from the rat and human have been the most extensively characterised (Sussenbach, 1989; Rotwein, 1991), although data from species such as the sheep and pig, are also available (Dickson *et al.*, 1991; Weller *et al.*, 1993).

1.1.3 IGF ACTIONS

The IGFs exert classical insulin-like effects on insulin-responsive tissues and cell types *in vitro*. For example, IGFs stimulate glucose uptake, lipid and glycogen synthesis and inhibit lipolysis in adipocytes, while in skeletal and cardiac muscle they stimulate

glucose uptake, glycolysis and glycogen synthesis (Froesch *et al.*, 1985). The IGFs also have anabolic and mitogenic, or growth promoting, actions *in vitro*. IGFs stimulate DNA and protein synthesis and inhibit protein degradation in various cell types, such as chick embryo fibroblasts, rat myoblasts (Ballard *et al.*, 1986) and bone cells (Froesch *et al.*, 1985). The IGFs may exert their mitogenic effects on cells through their action as progression factors in the cell cycle, stimulating the G_1 / G_0 to S-phase transition in actively growing cells such as fibroblasts (Froesch *et al.*, 1985; Cohick and Clemmons, 1993). The IGFs also promote differentiation of cultured cells such as rat myoblasts (Froesch *et al.*, 1985; Florini *et al.*, 1991).

These *in vitro* insulin-like, growth promoting and anabolic actions of IGFs can be demonstrated in the whole animal. Bolus injection of IGF-I or IGF-II into normal rats has a hypoglycaemic effect (Froesch *et al.*, 1985) while IGF-I administration to diabetic rats also lowers blood glucose (Hizuka *et al.*,1987). IGF-I and IGF-II stimulate increases in organ weights, body weight and body length in rodents (Schoenle *et al.*, 1985; Froesch *et al.*, 1985; van Buul-offers *et al.*, 1986) and promote nitrogen retention in catabolic states, such as that associated with glucocorticoid exposure in rats (Ballard *et al.*, 1991). These anabolic effects can also be demonstrated in larger mammalian species, such as the sheep (Douglas *et al.*, 1991). The major actions of IGF are summarised in Table 1.1.

Although the IGFs were originally considered to be produced by the liver and act as endocrine hormones, it is now apparent that the biological actions of IGF are also exerted through autocrine and paracrine actions of locally produced IGFs on target tissues (Holly and Wass, 1989; Le Roith and Roberts, 1991; Cohick and Clemmons, 1993).

1.1.4 IGF RECEPTORS

The mitogenic and metabolic actions of IGFs are mediated through interactions with two different kinds of receptor : the type I IGF receptor and the insulin receptor (Rechler and Nissley, 1985; Rechler and Nissley, 1986; Nissley and Lopaczynski, 1991). The type I IGF and insulin receptors have a high degree of structural homology, and are composed of a heterotetramer of two alpha and two beta chains, with intracellular tyrosine kinase activity

A. MAJOR ACTIONS OF IGF

Promote growth	via	↑ DNA synthesis
Mitogenic effects		↑ Protein synthesis
Anabolic effects		↓ Protein degregation
Metabolic effects (insulin-like)	—	↑ Glucose uptake

B. MAJOR REGULATORS OF IGF ACTION

MECHANISM	FACTOR	EFFECT
Production of protein or mRNA	Nutrition	↓
	GH (IGF-I only)	↑
	Cortisol	↓
	Others	↑↓
	Developmental status (1.3.2)	
Modulation of bioactivity	IGFBPs	↑↓

TABLE 1.1. SUMMARY OF THE MAJOR ACTIONS AND REGULATORS OF IGF. The actions and effectors of IGF action are described in more detail in 1.1.3 and 1.1.5, respectively. Arrows indicate a positive (up) or negative (down) regulation. Dual effects are indicated by both arrows.

(Figure 1.3). The IGF or insulin peptides bind with a much lower affinity to the heterologous receptor compared to their affinities for the homologous receptor (Nissley and Lopaczynski, 1991). The contribution of this receptor cross-reactivity to the actions of either insulin or IGF is unclear although it has been suggested that IGF can exert insulin-like actions independent of the insulin receptor (Ballard *et al.*, 1994). The IGFs also interact with a third receptor : the type II IGF receptor, which is identical to the cation-independent mannose-6-phosphate receptor, involved in lysosomal enzyme sorting and intracellular degradation pathways (Nissley and Lopaczynski, 1991). The type II IGF receptor binds IGF-II with high affinity, with little or no binding of IGF-I, and is present in both membrane bound and circulating forms (Nissley and Lopaczynski, 1991). It bears no structural homology to the type I IGF or insulin receptors (Figure 1.3) and its role in IGF signalling is unclear (Nissley and Lopaczynski, 1991). A recent hypothesis suggests that the type II IGF receptor acts to regulate IGF-II availability throughout fetal development by acting as an IGF-II 'sink' (Haig and Graham, 1991) which is further discussed in 1.3.2.2.

1.1.5 DISTRIBUTION AND REGULATION OF IGF PRODUCTION

The IGFs are produced by many cell and tissue types although the liver may be the primary source of circulating IGF-I (Daughaday and Rotwein, 1989; Cohick and Clemmons, 1993). In the adult rat, IGF-I mRNA and protein is present in many tissues such as the liver, kidney, heart and muscle, while, in contrast, IGF-II mRNA is abundant in only a few tissues, including the brain and kidney (Murphy *et al.*, 1987; Cohick and Clemmons, 1993). IGF-II mRNA is also restricted to the brain, liver, kidney and adrenal of the adult sheep (Delhanty and Han, 1993). Within these tissues the IGFs may be produced by a specific subset of cells. For example, in the rat ovary IGF-I mRNA is produced exclusively by granulosa cells, while IGF-II mRNA localises only to the thecal-interstitial cells (Hernandez *et al.*, 1989; Hernandez *et al.*, 1990). Thus, there is strict tissue and cell-type specific regulation of IGF production. The IGFs are also developmentally regulated (1.3.2).

Multiple mRNA transcripts are produced for both IGF-I and IGF-II through differential promoter usage and mRNA processing (1.1.2). The levels of these transcripts

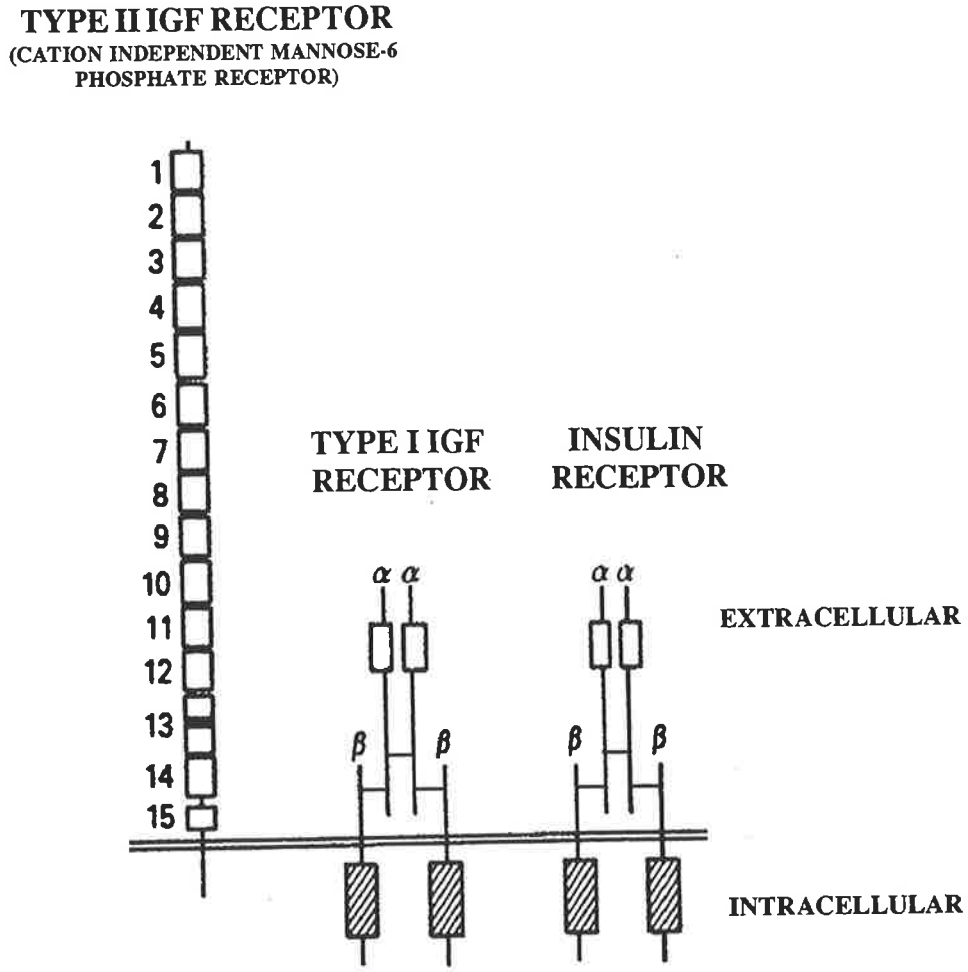


FIGURE 1.3. THE INSULIN-LIKE GROWTH FACTOR RECEPTORS. Schematic representations of the structures of the type I and type II IGF and insulin receptors are shown. Boxed regions in the type II IGF receptor indicate repeat sequences. Boxed regions in the type I IGF and insulin receptors indicate cysteine rich regions, while hatched boxes indicate the intracellular tyrosine kinase domains. Disulphide bonds joining the subunits within the type I IGF and insulin receptors are shown. (Adapted from Nissley and Lopacynski, 1991)

are regulated *in vivo* by nutritional, metabolic and hormonal factors (Rotwein, 1991; Cohick and Clemmons, 1993; Thissen *et al.*, 1994). The major regulator of circulating IGF-I levels, post-natally, is growth hormone (GH), as was indicated by the original observations of Salmon and Daughaday (1957), describing low somatomedin activity in serum from hypophysectomised rats. Circulating IGF-I levels are positively associated with GH levels and growth rates in many species such as rats and humans, and are elevated or decreased in altered growth states such as Laron dwarfism or acromegaly (Hall and Tally, 1989). Hepatic IGF-I mRNA levels are elevated in response to GH in many species, including the rodent and the sheep, although GH does not appear to stimulate IGF-I mRNA levels in non-hepatic tissues (Mathews *et al.*, 1986; Roberts *et al.*, 1986; Rotwein, 1991; Pell *et al.*, 1993). In contrast to the strong GH dependency of IGF-I, circulating IGF-II levels are not associated with GH status and liver IGF-II mRNA appears to be GH independent (Hynes *et al.*, 1987; Hall and Tally, 1989; Mesiano *et al.*, 1989).

IGF-I and IGF-II protein and mRNAs are regulated by many other endocrine factors. Epidermal growth factor (EGF) and parathyroid hormone (PTH) increase IGF-I while cortisol, chronic estrogen and interleukin-1 inhibit IGF-I (Daughaday and Rotwein, 1989; Rotwein, 1991; Lin *et al.*, 1992). The endocrine control of IGF-II is less well defined. However, cortisol or glucocorticoids are important negative regulators of IGF-II, reducing hepatic IGF-II mRNA in the rat or sheep *in vivo* (Beck *et al.*, 1988; Levinovitz and Norstedt, 1989; Li *et al.*, 1993) and IGF-II protein levels produced from cultured bone cells (Canalis *et al.*, 1991) .

One primary influence on IGF levels is nutrition (Rotwein, 1991; Thissen *et al.*, 1994). Fasting, malnutrition or restriction of protein intake reduces the levels of both circulating IGF-I and IGF-II in adult or neonatal rats (Elmer and Schalch, 1987; Phillips *et al.*, 1989; Donovan *et al.*, 1991), lambs (Pell *et al.*, 1993) and humans (Thissen *et al.*, 1994). Restricted nutritional intake also reduces hepatic IGF-I mRNA in species such as the sheep or rat (Pell *et al.*, 1993; Thissen *et al.*, 1994). The decrease in IGF protein and mRNA with nutritional restriction may be mediated via alterations in factors such as GH, insulin, triiodothyronine (T_3), or by changes in amino acid availability (Cohick and

Clemmons, 1993; Thissen *et al.*, 1994). The major regulators of IGF are summarised in Table 1.1.

Thus, regulation of IGF at the level of production is complex. Another major level of control of IGF action is through the interaction of IGFs with a family of proteins termed IGF-binding proteins (IGFBPs). The IGFBPs act to regulate IGF half-life, tissue delivery, tissue distribution and localisation of IGF and may both inhibit or stimulate IGF action (1.2.3). Therefore, the IGFBPs are critical modulators of the IGF axes.

1.2 THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

1.2.1 HISTORICAL ISOLATION OF IGFBPs

Initial characterisation of IGF in blood indicated that most of the circulating IGF activity existed as a high molecular weight form that was acid dissociable to low molecular weight IGF activity. This high molecular weight IGF binding activity was specific for IGF suggesting the existence of IGF binding species (Zapf *et al.*, 1975; Hintz and Liu, 1977). Subsequently, several IGF binding proteins (IGFBPs) were isolated from biological fluids, including amniotic fluid (Povoa *et al.*, 1984), placental extracts (Koistinen *et al.*, 1986), plasma (Martin and Baxter, 1986) and cell culture media from fetal rat fibroblasts, bovine kidney fibroblasts and human hepatocytes (Povoa *et al.*, 1985; Mottola *et al.*, 1986; Szabo *et al.*, 1988). N-terminal sequence analysis, protein characterisation and isolation of cDNA sequences for the purified IGFBPs indicated the existence of three different forms of IGFBPs. These were subsequently named IGFBP-1, IGFBP-2 and IGFBP-3 (Ballard *et al.*, 1990). Within one year of assigning the nomenclature for IGFBP-1, IGFBP-2 and IGFBP-3, further novel IGFBPs were purified from ovine, rat and human plasma (Walton *et al.*, 1990; Shimonaka *et al.*, 1989; Kiefer *et al.*, 1991a; Shimasaki *et al.*, 1991a), bone, neuroblastoma or lung fibroblast cell culture media (Mohan *et al.*, 1989; Forbes *et al.*, 1990; Andress and Birnbaum, 1991; Ceda *et al.*, 1991) and ovarian follicular fluid (Shimasaki *et al.*, 1991b). These new IGFBPs were termed IGFBP-4, IGFBP-5 and IGFBP-6 (Ballard *et al.*, 1992). Prior to this agreed nomenclature Kiefer *et al.* (1991a and b) cite IGFBP-4 as IGFBP-5, IGFBP-5 as IGFBP-6 and IGFBP-6 as IGFBP-4.

1.2.2 IGFBP PROTEIN AND GENE STRUCTURE

The IGFBPs comprise a family of at least six homologous proteins that are unrelated in sequence or structure to either the type I or type II IGF receptor (Figure 1.4 and 1.5). The sequences of the six known rat and human IGFBPs are shown in Figures 1.4 and 1.5 respectively, which illustrates the strong homology between the different IGFBPs. The protein sequence is conserved at both the N- and C-terminal ends with a central divergent region (Figure 1.4 and 1.5). There are 18 conserved cysteine residues, with the exception of rat IGFBP-6, which has 14 cysteines (Figure 1.4) and human IGFBP-6 which contains 16 cysteines (Figure 1.5). IGFBP-4 also has two additional cysteine residues in the central divergent region (Figure 1.4 and 1.5). The gene structures for each IGFBP have been determined in at least one species (Rechler, 1993). The position of the intron / exon boundaries are conserved throughout the IGFBP genes, suggesting divergence of the IGFBP family from a common ancestral gene (Rechler, 1993). Human IGFBP-1 and IGFBP-3 have been localised to the same region of chromosome 7 (Rechler, 1993). Human IGFBP-2 and IGFBP-5 both localise to chromosome 2 (Allander *et al.*, 1994), although Shimasaki *et al.* (1991a) suggest human IGFBP-5 to be located on chromosome 5.

1.2.2.1 IGFBP-1

IGFBP-1 has been previously termed the amniotic fluid binding protein, placental protein 12, α-1-pregnancy associated endometrial globulin and HEP G2 binding protein (Baxter and Martin, 1989; Ballard *et al.*, 1990). IGFBP-1 is a 25 to 28 kDa protein that binds IGF-I and IGF-II with approximately equal affinities and contains an Arg-Gly-Asp (RGD), fibronectin binding sequence, suggesting cell surface or matrix binding activity (Rechler, 1993; Baxter, 1993). Full cDNA sequences have been reported for human (Julkunen *et al.*, 1988; Brinkman *et al.*, 1988; Lee *et al.*, 1988; Grundmann *et al.*, 1988; Brewer *et al.*, 1988), rat (Murphy *et al.*, 1990), ovine (Phillips *et al.*, 1991b) and mouse IGFBP-1 (Schuller *et al.*, 1994) (Table 1.2). The mRNA for IGFBP-1 exists as a single species of approximately 1.5 to 1.6 kb and the human and rat genes have been isolated and characterised (Cubbage *et al.*, 1989; Unterman *et al.*, 1992) (Table 1.2). The human gene is

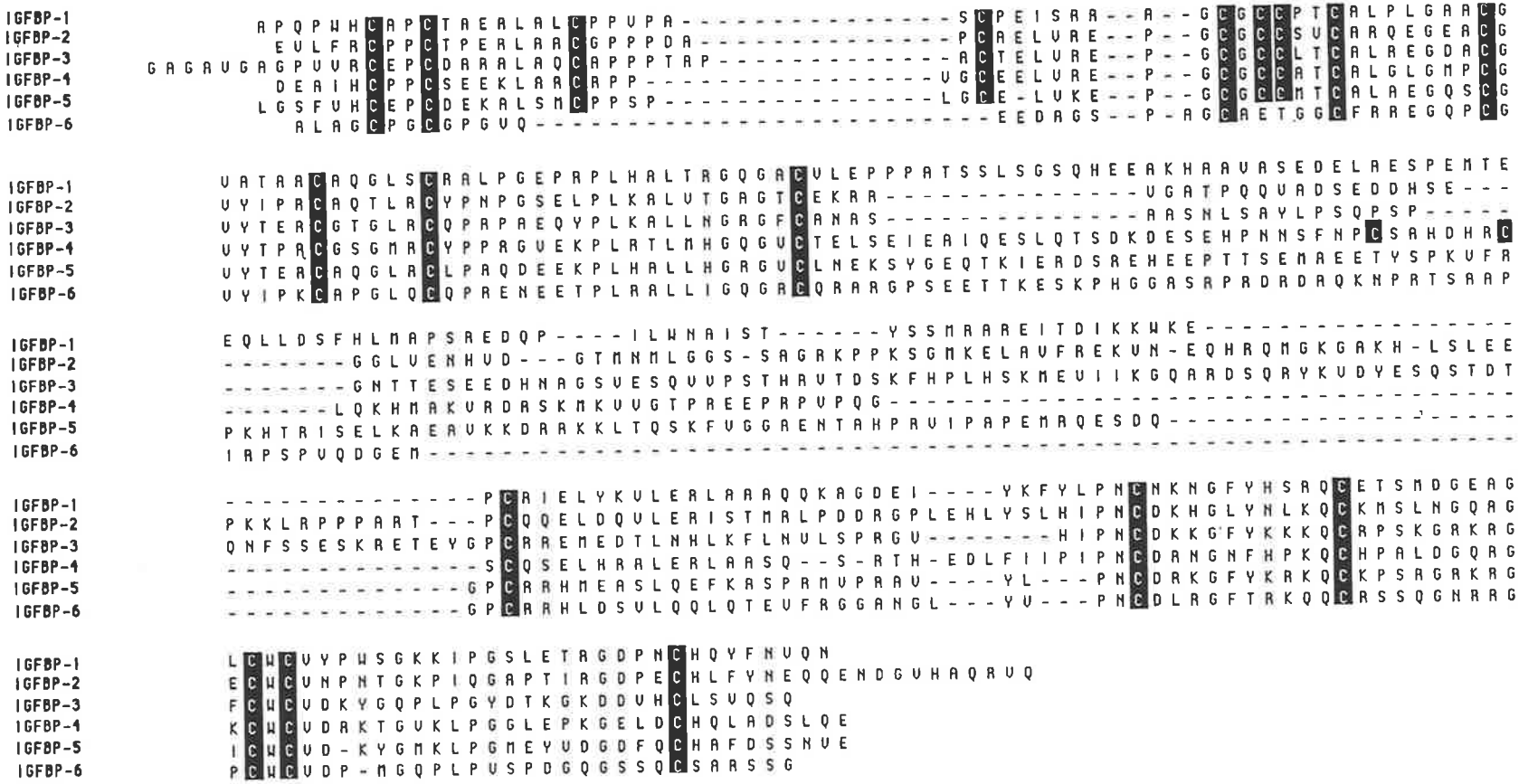


FIGURE 1.4 SEQUENCE ALIGNMENT OF RAT IGFBPs. Protein sequences of rat IGFBP-1 to IGFBP-6 are shown. The cysteine residues are highlighted by black boxes. Figures 1.4 and 1.5 were generated by Dr. B. E. Forbes. Note the conservation of the positions of the cysteine residues and the high sequence homology in the N- and C-terminal regions of the IGFBPs.

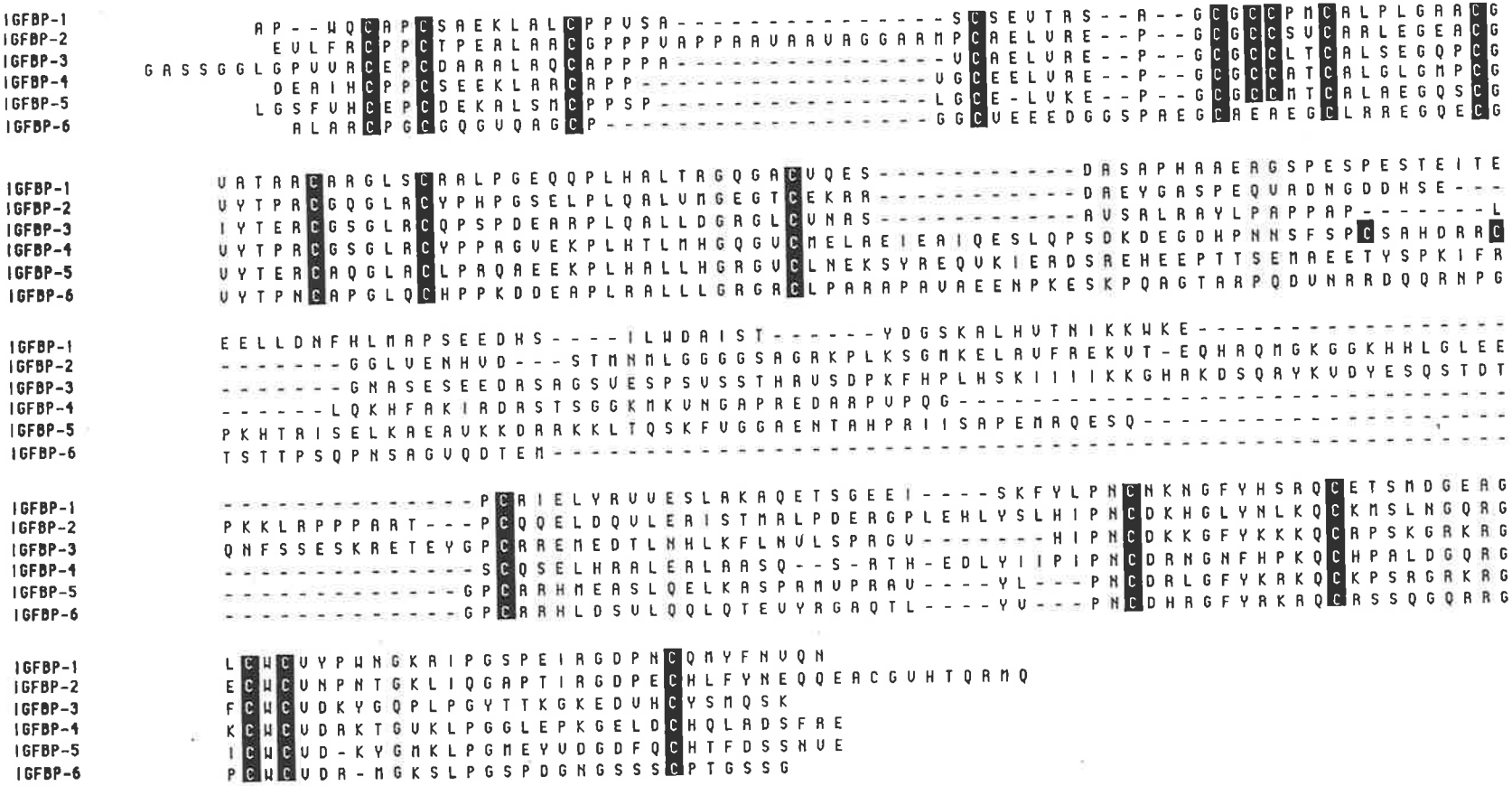


FIGURE 1.5 SEQUENCE ALIGNMENT OF HUMAN IGFBPs. Protein sequences of human IGFBP-1 to IGFBP-6 are shown. The cysteine residues are highlighted by black boxes.

IGFBP	SPECIES	cDNA REFERENCE	GENE REFERENCE
IGFBP-1	HUMAN	Julkunen *et al.*, 1988 Brinkman *et al.*, 1988 Lee *et al.*, 1988 Grundmann *et al.*, 1988 Brewer *et al.*, 1988	Cubbage *et al.*, 1989
	RAT	Murphy *et al.*, 1990	Unterman *et al.*, 1992
	OVINE	Phillips *et al.*, 1991b	
	MOUSE	Schuller *et al.*, 1994	
IGFBP-2	HUMAN	Binkert *et al.*, 1989	Brown and Rechler, 1990
	RAT	Margot *et al.*, 1989	Ehrenborg *et al.*, 1991
	OVINE	Delhanty and Han, 1993	
	MOUSE	Schuller *et al.*, 1994	Binkert *et al.*, 1992
	BOVINE	Upton *et al.*, 1990	
IGFBP-3	HUMAN	Wood *et al.*, 1988 Spratt *et al.*, 1990	Cubbage *et al.*, 1990
	RAT	Shimasaki *et al.*, 1989 Albiston and Herington, 1990	
	OVINE	Hayatsu *et al.*, 1994	
	MOUSE	Schuller *et al.*, 1994	
	BOVINE	Spratt *et al.*, 1991	
	PORCINE	Shimasaki *et al.*, 1990b	
IGFBP-4	HUMAN	Shimasaki *et al.*, 1990 Kiefer *et al.*, 1991a	Mohan *et al.*, 1994 Zazzi *et al.*, 1994
	RAT	Shimasaki *et al.*, 1990	Gao *et al.*, 1993
	OVINE	Carr *et al.*, 1994a (Chapter 3)	
	BOVINE	Moser *et al.*, 1992	
	MOUSE	Schuller *et al.*, 1994	
IGFBP-5	HUMAN	Kiefer *et al.*, 1991b	Allander *et al.*, 1994
	RAT	Shimasaki *et al.*, 1991a	Zhu *et al.*, 1993
	MOUSE	James *et al.*, 1993 Schuller *et al.*, 1994	Kou *et al.*, 1994
IGFBP-6	HUMAN	Kiefer *et al.*, 1991a	S. Shimasaki (pers. comm.,Rechler, 1993)
	RAT	Shimasaki *et al.*, 1991b	
	MOUSE	Schuller *et al.*, 1994	

TABLE 1.2 SUMMARY OF KNOWN cDNA AND GENE SEQUENCES FOR IGFBPs. Published cDNA and gene sequences for IGFBPs 1 to 6 from different species are cited. The gene for human IGFBP-6 has been isolated by S. Shimasaki and cited as personal communication by Rechler (1993).

approximately 5.2 kb in size containing four exons. The promoter regions contain TATA and CAAT boxes, an essential binding site for the transcription factor Hepatic Nuclear Factor-1 (HNF-1) and insulin and glucocorticoid responsive regions (Suwanichkul, *et al.*, 1993; Goswami *et al.*, 1994). IGFBP-1 has been suggested to play a major role in glucose homeostasis due to the insulin and glucose responsiveness of this protein *in vivo* and the insulin responsive regions of the human IGFBP-1 promoter (Lewitt and Baxter, 1991a and 1991b; Lee *et al.*, 1993b) (1.2.5.1).

1.2.2.2 IGFBP-2

IGFBP-2 has been previously termed the BRL-3A and MDBK binding protein, is a 29 to 30 kDa non-glycosylated protein, with markedly preferential binding of IGF-II in comparison to IGF-I (Baxter and Martin, 1989; Ballard *et al.*, 1990; Baxter, 1993). As for IGFBP-1, IGFBP-2 contains an RGD sequence and is represented by a single mRNA transcript of 1.4 to 1.6 kb (Baxter, 1993; Rechler, 1993). cDNA sequences have been presented for human (Binkert *et al.*, 1989), rat (Margot *et al.*, 1989), ovine (Delhanty and Han, 1993), bovine (Upton *et al.*, 1990) and mouse (Schuller *et al.*, 1994) (Table 1.2). IGFBP-2 genes have been isolated from the human, rat and mouse and are 30 to 40 kb in size, primarily due to a large intron 1 (27 to 35 kb) (Brown and Rechler, 1990; Ehrenborg *et al.*, 1991; Binkert *et al.*, 1992) (Table 1.2). The IGFBP-2 gene contains four exons and the promoter region has no identifiable TATA or CAAT elements, but is highly GC rich with several direct and indirect repeat sequences (Brown and Rechler, 1990; Binkert *et al.*, 1992). IGFBP-2 levels are elevated in fetal serum in comparison to the adult in species such as the rat, sheep, primate and pig and thus may be important for fetal growth and development (Donovan *et al.*, 1989; Butler and Gluckman, 1986; Liu *et al.*, 1991; Lee *et al.*, 1993a) (1.3.3.1).

1.2.2.3 IGFBP-3

IGFBP-3 has previously been cited as the acid stable IGFBP or the GH-dependent IGFBP and binds IGF-I and IGF-II with approximately equal affinities (Baxter and Martin,

1989; Ballard *et al.*, 1990; Baxter, 1993). IGFBP-3 is a 28 kDa core protein that is N-glycosylated to form species of apparent molecular weights between 42 to 55 kDa, typically present as a doublet on non-reducing SDS-PAGE (Baxter, 1993; Rechler, 1993). IGFBP-3 cDNA sequences have been reported from human (Wood *et al.*, 1988; Spratt *et al.*, 1990), rat (Shimasaki *et al.*, 1989; Albiston and Herington, 1990), ovine (Hayatsu *et al.*, 1994), bovine (Spratt *et al.*, 1991), porcine (Shimasaki *et a*l., 1990b) and mouse (Schuller *et al.*, 1994) (Table 1.2). IGFBP-3 mRNA is represented by a single transcript of approximately 2.3 to 2.6 kb. The human IGFBP-3 gene has been isolated and is approximately 9 kb and contains five exons (Cubbage *et al.*, 1990). The additional exon compared to the other IGFBP genes comprises 3' untranslated sequences, again showing the conservation of the intron / exon boundaries across the coding regions of IGFBPs. The promoter region of human IGFBP-3 has been identified and contains both TATA and GC boxes. IGFBP-3 is the major circulating IGFBP in adult mammals and exists primarily as a high molecular weight (150 kDa) ternary complex. This complex is comprised of IGFBP-3, IGF-I or IGF-II and a third non-IGF binding protein termed the acid labile subunit (ALS) (Baxter, 1993; Rechler, 1993) (1.2.2.7).

1.2.2.4 IGFBP-4

At the time of commencement of this project, collaborators P. E. Walton and P. A. Grant, Co-operative Research Centre (CRC) for Tissue Growth and Repair, had isolated an IGFBP from ovine plasma with novel N-terminal sequence that was relatively abundant in the adult sheep and present as two different molecular weight forms (Walton *et al.*, 1990). This novel IGFBP, of unknown physiology or molecular biology, represented the ovine equivalent of other novel IGFBPs purified from human bone cell culture medium, human and rat plasma and a neuroblastoma cell line and termed IGFBP-4 (Ballard *et al.*, 1992). IGFBP-4 is a 25 kDa protein, that also exists as a 29 kDa form due to N-linked glycosylation, and binds both IGF-I and IGF-II with approximately equal affinities (Baxter, 1993; Rechler, 1993). The major mRNA transcript for IGFBP-4 is approximately 2.6 kb, with two smaller transcripts of 1.8 to 2.1 kb reported in some studies in both human and rat

cells (Ceda *et al.*, 1991; Camacho-Hubner *et al.*, 1992) and in this thesis, in the sheep (Chapter 3; Carr *et al.*, 1994a). cDNA sequences for IGFBP-4 have been reported from human, rat (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a), bovine (Moser *et al.*, 1992) and mouse (Schuller *et al.*, 1994). Chapter 3 presents sequence data for ovine IGFBP-4, which is the first sequence data available for IGFBP-4 in this species (Carr *et al.*, 1994a) (Table 1.2). The genes for human and rat IGFBP-4 have recently been isolated (Gao *et al.*, 1993; Mohan *et al.*, 1994; Zazzi *et al.*, 1994) (Table 1.2). The rat gene is at least 12 kb in size with four exons and contains TATA and CAAT boxes, cAMP responsive elements and a putative progesterone receptor element in the promoter region (Gao *et al.*, 1993).

1.2.2.5 IGFBP-5

IGFBP-5 has been purified from human serum and human bone extracts, where it represents the major IGFBP (Kiefer *et al.*, 1991b; Bautista *et al.*, 1991). The protein is approximately 29 kDa and shows no potential glycosylation site or RGD sequence, but binds with high affinity to hydroxyapatite and thus bone matrix (Bautista *et al.*, 1991). IGFBP-5 binds IGF-II with higher affinity than IGF-I (Baxter, 1993; Rechler, 1993) and cDNA sequences for IGFBP-5 have been reported for human (Kiefer *et al.*, 1991b), rat (Shimasaki *et al.*, 1991a) and mouse (James *et al.*, 1993; Schuller *et al.*, 1994) (Table 1.2). IGFBP-5 shows an mRNA transcript of approximately 6 kb (Rechler, 1993). The genes for rat, human and mouse IGFBP-5 have been isolated and vary in size from 17 to 33 kb, but again all contain four exons (Zhu *et al.*, 1993; Allander *et al.*, 1994; Kou *et al.*, 1994) (Table 1.2). Functional promoters have been identified, which in the rat contain TATA and CAAT boxes as well as putative Activator Protein-1 (AP-1), AP-2 and progesterone receptor binding elements (Zhu *et al.*, 1993).

1.2.2.6 IGFBP-6

IGFBP-6 has been purified from fluids such as human lung fibroblast cell culture media, and human and rat serum (Ballard *et al.*, 1992). The core protein is predicted to be 22 to 23 kDa, smaller than the reported size of 28 to 34 kDa when isolated from various

sources due to both N- and O-linked glycosylation (Baxter, 1993; Rechler, 1993). IGFBP-6 shows marked preferential binding of IGF-II over IGF-I (Baxter, 1993; Rechler, 1993). The mRNA for IGFBP-6 is approximately 1.3 kb in size and cDNA sequences have been characterised in the human (Kiefer *et al.*, 1991a), rat (Shimasaki *et al.*, 1991b) and mouse (Schuller *et al.*, 1994) (Table 1.2). Human IGFBP-6 lacks the sixth and seventh cysteines, while rat IGFBP-6 lacks the third, fourth, sixth and seventh cysteine residues common to all other IGFBPs (Figure 1.4 and 1.5). The gene for rat IGFBP-6 has been isolated and reported by S. Shimasaki in personal communication (Rechler, 1993). The gene is of undetermined size, with four exons and no identifiable TATA box.

1.2.2.7 Acid labile subunit (ALS)

IGFBP-3 exists in the circulation, mainly as a high molecular weight ternary complex of approximately 150 kDa that is formed by association with IGF and an acid labile subunit (ALS). The ALS is a core protein of approximately 60 kDa and is N-glycosylated to a molecular weight of approximately 90 kDa. The ALS is unstable at pH < 5 (Baxter, 1993; Rechler, 1993). cDNA sequences for human and rat ALS have been isolated and show distinctive leucine rich repeat domains in the translated protein, characteristic of regions involved in protein - protein interactions (Leong *et al.*, 1992; Dai and Baxter, 1992). The ALS does not bind IGF or IGFBP-3 without IGF, indicating that formation of the 150 kDa complex occurs in an ordered fashion with initial binding of IGF to IGFBP-3 (Baxter, 1993). Circulating concentrations of ALS in plasma are reported to be in excess of those for IGFBP-3 on a molar basis, are GH dependent, and negatively regulated by EGF and glucocorticoids (Baxter, 1993; Dai *et al.*, 1994).

Human or bovine IGFBP-1 to IGFBP-6 have been produced recombinantly in either yeast or *E. coli* (Sommer *et al.*, 1993; Kiefer *et al.*, 1992; Forbes *et al.*, 1994) and antibodies to many IGFBPs have been produced (Liu *et al.*, 1993b) and are commercially available. Specific radioimmunoassays to measure IGFBPs and the ALS have been developed in many species, including the rat, human, pig and sheep (Rutanen *et al.*, 1982;

McCusker *et al.*, 1991; Baxter and Martin, 1986; Baxter and Saunders, 1992; Baxter and Dai, 1994; Gallaher *et al.*, 1994). However, most available antibodies for IGFBP-4 cross-react with IGFBP-2 and thus specific radioimmunoassays for IGFBP-4 are not readily available (Chapter 4). The availability of antibodies, large amounts of IGFBP protein, IGFBP assays and cDNA probes, provides useful tools for the future analysis of IGFBP structure, function and regulation.

1.2.3 THE ACTIONS OF IGFBPs

The IGFBPs have been shown to play several important roles in determining the actions of the IGFs (Table 1.3). Recently IGFBP-3 and IGFBP-5 have been suggested to have intrinsic biological activity (Bar *et al.*, 1994, Rosenfeld, 1994) although evidence supporting independent actions of IGFBPs is still questionable and, to maintain brevity and focus in this review, will not be discussed further.

1.2.3.1 Increased circulating half-life of IGF

The association of IGF with IGFBPs increases the circulating half-life of IGF. Cohen and Nissley (1976) first suggested this upon observation that the half-life of somatomedin was decreased in hypophysectomised rats and restored upon GH treatment. Subsequent investigations in the rat and sheep have demonstrated that the increased half-life of circulating IGF is primarily due to the association of IGF-I or IGF-II into the 150 kDa complex, although IGF bound to the lower molecular weight IGFBPs also has an extended half-life in comparison to free IGF (Zapf *et al.*, 1986; Hodgkinson *et al.*, 1989).

1.2.3.2 Tissue delivery, distribution and localisation of IGF

The IGFBPs can transverse capillary barriers and show selective tissue localisation. Bar *et al.* (1990a) have shown, in the isolated perfused rat heart, that IGFBP / IGF-I conjugates cross the capillary endothelium and preferentially localise to cardiac muscle. In the same system, IGFBPs -1, -2, -3 and -4, alone, all cross capillary barriers, although IGFBP-1, -2 and -3 subsequently localise to cardiac muscle and IGFBP-4 localises to

A. MAJOR ACTIONS OF IGFBPs

- ⬆ Half-life of circulating IGF
- Alteration of IGF tissue delivery and distribution
- Localisation of IGF within specific organs
- Modulation of IGF action ⬆ or ⬇

B. MAJOR REGULATORS OF IGFBP ACTIONS

MECHANISM	FACTOR	EFFECT
Production of protein or mRNA	IGF	⬆⬇
	Nutrition	⬇⬆
	Insulin (IGFBP-1 and IGFBP-2)	⬇
	Thyroid Hormones	⬆⬇
	Glucorcorticoids / cortisol	⬆⬇
	Others	⬆⬇
	Developmental age (1.3.3)	
Alteration of protein levels	IGFBP proteases	⬇
Alteration of IGF binding affinity	IGFBP proteases	⬇
	Phosphorylation	⬆

TABLE 1.3. SUMMARY OF THE MAJOR ACTIONS AND REGULATORS OF IGFBPs. The actions of IGFBPs and regulators of IGFBPs are described in more detail in 1.2.3 and 1.2.5, respectively. Arrows indicate positive (up) or negative (down) regulation. Both arrows indicate dual effects on different IGFBPs or the same IGFBP under different conditions.

connective tissue (Boes *et al.*, 1992). This selective tissue localisation can be affected by hormones such as insulin (Bar *et al.*, 1990b). IGFBP-1 and -2 administered to rats rapidly leaves the circulation and equilibrates with extravascular compartments (Young *et al.*, 1992). This re-distribution of circulating IGFBPs has been hypothesised to modulate the transvascular transport of IGF-I and IGF-II (Young *et al.*, 1992). In contrast to this, the 150 kDa complex is suggested to be restricted to the circulation due to the size permeability constraint imposed by the vascular endothelium. IGFBPs may also localise IGFs to particular sites within individual systems and serve as storage pools for IGF. For example, IGFBP-5 binds extensively to bone matrix and has been proposed to localise IGF-II within bone (Bautista *et al.*, 1991). It is hypothesised that during bone remodelling the matrix is digested, releasing IGF-II and IGFBP-5, which are subsequently involved in the bone remodelling process (Bautista *et al.*, 1991). *In situ* analysis, of organs such as the kidney, ovary and lung, demonstrate specific localisation of different IGFBPs within the same tissue, often with specific co-localisation of either IGF-I or IGF-II (Nakatani *et al.*, 1991; Chin *et al.*, 1992; Klempt *et al.*, 1992). Thus, different IGFBPs may be involved in determining IGF tissue delivery, distribution and localisation, ultimately affecting the site of IGF action.

1.2.3.3 Modulation of IGF action

The binding of IGFs by IGFBPs can modulate IGF action. Initially the IGFBPs were considered inhibitors of IGF activity. Bovine IGFBP-2 inhibits IGF stimulated DNA and protein synthesis and IGF inhibition of protein degradation in chick embryo fibroblasts, but is less effective in inhibiting the actions of IGF analogues that do not bind as well as IGF-I or IGF-II to IGFBP-2 (Ross *et al.*, 1989). Porcine IGFBP-3 can block the insulin-like activities of IGFs on adipose tissue (Walton *et al.*, 1989), while both IGFBP-1 and -3 have been shown to inhibit IGF stimulated ovarian granulosa cell steroidogenesis in rat and human cell cultures (Ui *et al.*, 1989; Mason *et al.*, 1992). However, IGFBP inhibition of IGF action is not observed in all instances. A binding protein isolated from human amniotic fluid (presumably IGFBP-1) has been shown to potentiate the actions of IGF-I on fibroblast and muscle cells from human, mouse, chicken and pig (Elgin *et al.*, 1987), while Blum *et al.*

(1989) have demonstrated enhancement of IGF-I stimulated DNA synthesis in hamster kidney or human skin fibroblasts when complexed to an IGFBP purified from human plasma. Many investigators have shown both inhibition and potentiation of IGF action by IGFBPs in the same experimental system. Pre-incubation of human neonatal fibroblast cultures with IGFBP-3 potentiates IGF-I stimulated DNA synthesis, but co-addition of both factors inhibits these effects of IGF-I (De Mellow and Baxter, 1988). IGFBP-1 purified from human placenta potentiates IGF stimulated DNA synthesis in fibroblasts, although the same IGFBP-1 preparation inhibits these actions in granulosa cells (Koistinen *et al.*, 1990). Soluble bovine IGFBP-3 inhibits IGF stimulated amino acid uptake in bovine fibroblasts, but pre-incubation of cells with IGFBP-3 leads to cell surface association of this binding protein and enhancement of IGF action (Conover *et al.*, 1990). Further experiments have suggested that this enhancement of IGF action is due to proteolysis of cell associated IGFBP-3 to a form with lower affinity for IGF (Conover, 1992). Schmid *et al.* (1991) have also shown that intact but not truncated IGFBP-3 inhibits IGF actions in osteoblasts. Recently, Jones *et al.* (1993) have shown that extracellular matrix associated IGFBP-5, but not soluble IGFBP-5, potentiates the stimulatory action of IGF-I on cell number in human fetal fibroblasts. Therefore, the effects of IGFBPs on the actions of IGFs are dependent upon the type of IGFBP, its modification by post-translational events, the cell or tissue type and its previous exposure to growth factors.

1.2.3.4 Functions of IGFBPs

Although the IGFBPs have been shown to have many actions, the biological roles of specific IGFBPs are largely unknown. IGFBP-5 has been suggested to be involved in bone remodelling (Bautista *et al.*, 1991), IGFBP-2 in fetal growth and development (1.3.3) and IGFBP-2, -3 and -4 have been suggested to function in ovarian follicle development (Samaras *et al.*, 1992; Erickson *et al.*, 1992a and 1992b; Monget *et al.*, 1993). However, the most convincing evidence for a specific role of any IGFBP is that for the involvement of IGFBP-1 as a glucose counter-regulatory hormone (Lewitt and Baxter, 1991a and 1991b; Lee *et al.*, 1993b). This is suggested by both *in vivo* and *in vitro* data and the

structure of the IGFBP-1 promoter. IGFBP-1 is negatively regulated by insulin (1.2.5.1) while the insulin and glucocorticoid responsive elements of the IGFBP-1 promoter are structurally similar to promoter elements of the phospho-enol pyruvate carboxy kinase gene (PEPCK), which encodes an insulin responsive enzyme, involved in glucose metabolism (Suwanichkul *et al.*, 1993). Direct evidence for a role of IGFBP-1 in glucose regulation comes from the observation that infusion of IGFBP-1 into rats blocks IGF-I induced hypoglycaemia, while IGFBP-1 administered alone elevates blood glucose (Lewitt *et al.*, 1991a).

1.2.4 DISTRIBUTION OF IGFBPs

The IGFBPs are produced by many different cell and tissue types and are present in biological fluids, such as plasma, lymph, amniotic fluid, milk and urine (Gargosky *et al.*, 1990b; Rechler, 1993). IGFBP-1, IGFBP-3 and IGFBP-6 are produced by most tissues of the adult including the liver, kidney, lung, stomach, heart and placenta, in the rat or human, with varying abundance of IGFBP mRNA levels in different tissues (Murphy *et al.*, 1990; Albiston and Herington, 1990; Shimasaki *et al.*, 1991b). The distribution of IGFBP-4 mRNA has only been studied in adult rat tissues and human or bovine cell lines, which indicates wide spread expression, predominating in the liver (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a). In contrast to this wide distribution of mRNA for IGFBP-1, -3, -4, and -6, IGFBP-2 mRNA in the adult rat or sheep is restricted, as for IGF-II, primarily to the brain and kidney (Margot *et al.*, 1989) and also the liver in the sheep (Delhanty and Han, 1993). For IGFBPs 1 to 4 and IGFBP-6, mRNA levels in the liver are abundant relative to most other tissues (Rechler, 1993). However, mRNA for IGFBP-5 differs to that of the other IGFBPs, in that the IGFBP-5 mRNA transcript is much larger than those for other IGFBPs (6 kb) and is low in the liver and more abundant in tissues such as the kidney, lung, heart, stomach and intestine (Shimasaki *et al.*, 1991b; Rechler, 1993). Therefore, the different IGFBPs show some elements of common mRNA distribution but also display tissue specific expression.

1.2.5 REGULATION OF IGFBPs

Like the IGFs, the IGFBPs are regulated by nutritional, hormonal and metabolic factors, as is summarised in Table 1.3 (Rechler, 1993; Baxter, 1993; Thissen *et al.*, 1994). These factors can regulate IGFBP production, IGFBP degradation by specific proteolysis, or IGFBP binding affinity for IGFs, by proteolytic cleavage or post-translational modification.

1.2.5.1 Regulation of IGFBP production

Regulation by GH and IGF

Serum somatomedin or IGF binding activity was observed to be GH dependent due to the low binding activity of hypophysectomised rat serum compared to normal (Moses *et al.*, 1976). A GH-dependent IGFBP was isolated and later termed IGFBP-3 (Ballard *et al.*, 1990). However, further experiments in the rat have shown that, although the formation of the 150 kDa complex is GH dependent due to GH regulation of ALS, the production of IGFBP-3 is related to GH via direct regulation by IGF-I (Baxter, 1993). Measurements of IGF-I and IGFBP-3 mRNA levels in the liver of hypophysectomised rats indicates that changes in IGFBP-3 mRNA levels, induced by hypophysectomy or GH replacement, are associated with major changes in hepatic IGF-I mRNA abundance (Albiston and Herington, 1992). IGF-I levels are strongly associated with IGFBP-3 levels in many physiological situations (Baxter, 1988). Direct evidence for the IGF dependence of IGFBP-3 comes from several studies, whereby infusions of IGF-I into hypophysectomised rats restored serum IGFBP-3 levels (Clemmons *et al.*, 1989; Zapf *et al.*, 1989), and through interbreeding of IGF-I transgenic and GH deficient dwarf mice and subsequent comparison of circulating IGFBP levels (Camacho-Hubner *et al.*, 1991). This latter study further showed that IGF-I overexpressing mice also have elevated levels of circulating IGFBP-2. Stimulation of IGFBP-2 by IGFs has been suggested by several other studies, although a concomitant decline in insulin levels may be the direct mechanism responsible for these effects (Rechler, 1993). IGF-I or IGF-II have been shown to stimulate IGFBPs, at both the protein and mRNA level, from many cell types such as cultured human or rat fibroblasts (Hill *et*

al.,1989; Martin and Baxter, 1990), rat pituitary cells (Simes *et al.*, 1991), rat osteoblasts (Schmid *et al.*, 1989), bovine mammary epithelial cells (McGrath *et al.*, 1991) and rat astroglial cells (Bradshaw and Han, 1993). However, the effect of IGF on IGFBP production is not always stimulatory. For example, IGF-I inhibits the release of IGFBP-1 from cultured human decidual cells (Thrailkill *et al.*, 1990), stimulates IGFBP-4 production in human epidermal squamous cell carcinomas, but inhibits IGFBP-4 production in human skin fibroblasts (Neely and Rosenfeld, 1992). Thus, the regulation of IGFBP production by IGF is cell type and tissue specific.

Regulation by nutrition

Nutritional and metabolic factors are important regulators of IGFBPs (Thissen *et al.*, 1994). Fasting or protein deprivation increases circulating IGFBP-1 and IGFBP-2 and decreases plasma IGFBP-3 in fetal sheep (Gallaher *et al.*, 1992), pigs (McCusker *et al.*, 1989), rats and humans (Thissen *et al.*, 1994). However, the effects of nutrition on circulating IGFBP-2 and IGFBP-3 are often only observed in chronic dietary manipulations such as long term fasting of sheep or anorexia in humans (Cohick and Clemmons, 1993; Rechler, 1993; Thissen *et al.*, 1994). Nutritional regulation of IGFBPs may be mediated through changes in circulating glucose and insulin levels, as well as factors such as GH, IGF and T_3 (Rechler, 1993; Thissen *et al.*, 1994).

Regulation by insulin

The regulation of IGFBP-1 by insulin has been an area of intense research. IGFBP-1 has been suggested to play an important role in regulation of glucose levels (Lewitt and Baxter, 1991b; Lee *et al.*, 1993b) (1.2.3.4). Several studies have shown IGFBP-1 to undergo diurnal variation in the human, with IGFBP-1 levels being highest in the morning. This rhythm is not an intrinsic control mechanism and is related to food intake (Baxter, 1993; Lee *et al.*, 1993b). IGFBP-1 is inversely related to glucose levels in many situations in the rat or human, being elevated in hypoglycaemia and inhibited by high glucose and high insulin. However, hyperinsulinaemia with euglycaemia in humans, is associated with a

reduction in circulating IGFBP-1, suggesting that IGFBP-1 is primarily regulated by insulin rather than glucose (Brismar *et al.*, 1988; Suikkari *et al.*, 1989). The IGFBP-1 promoter has an insulin responsive region through which this regulation is probably mediated (Suwanichkul *et al.*, 1993). IGFBP-2 protein and mRNA is also inhibited by insulin in humans and rats (Boni-Schnetzler *et al.*, 1990; Schmid *et al.*, 1992a; Neely and Rosenfeld, 1992; Rechler, 1993). IGFBP-1 and IGFBP-2 mRNAs are both increased in streptozotocin diabetic rats, through altered transcription for IGFBP-1, but primarily via post-transcriptional mechanisms for IGFBP-2 (Ooi *et al.*, 1992). The increased levels of IGFBP-1 and IGFBP-2 in the streptozotocin diabetic rat are normalised by insulin replacement, although the time course for down regulation of IGFBP-1 and IGFBP-2 mRNAs by insulin are different, with IGFBP-1 mRNA being rapidly reduced and IGFBP-2 mRNA decreasing slowly (Ooi *et al.*, 1992). Thus, Ooi *et al.* (1992) hypothesised that these differences in the mechanisms of insulin regulation of IGFBP-1 and IGFBP-2 may reflect a role for IGFBP-1 in acute and IGFBP-2 in chronic adaptations to metabolic change.

Regulation by the thyroid gland, glucocorticoids and other factors

The IGFBPs are regulated by many hormones not discussed herein. Studies of the effects of hypophysectomy on IGFBPs in the rat demonstrate the importance of the hypothalamus and pituitary in regulating IGFBP levels post-natally (Rechler, 1993). However, the effect of hypophysecotmy on circulating IGFBPs is not only due to the loss of the GH / IGF axes, but also involves the disruption of other endocrine axes. One endocrine gland important in regulating IGF and IGFBP production (and influenced by hypophysectomy) is the thyroid. Parathyroid hormone (PTH) and T_3 stimulate IGFBP-2 and IGFBP-3 mRNA and protein levels in rat osteoblasts or pituitary cells (Schmid *et al.*, 1992b; Ceda *et al.*, 1992), while PTH can also stimulate IGFBP-4 and IGFBP-5 in rat osteoblasts (Torring *et al.*, 1991; Conover *et al.*, 1993b). Congenitally hypothyroid rats have increased IGFBP-2 levels while adult hypothyroid rats show no change in IGFBP-2, but have low IGFBP-3 and IGFBP-4 levels, indicating regulation of circulating IGFBPs by the thyroid occurs in a developmentally specific manner (Nanto-Solonen and Rosenfeld,

1992). The sheep thyroid follicle is responsive to both IGFs and IGFBPs and IGFBP production is inhibited by Thyroid Stimulating Hormone (TSH) (Phillips *et al.*, 1994). These results suggest that the regulation and interactions of IGFBPs by the thyroid is complex.

The levels of IGFBPs are also influenced by glucocorticoids. Dexamethasone increases IGFBP-1 protein and mRNA in the rat *in vivo* or *in vitro* (Luo *et al.*, 1990; Orlowski *et al.*, 1990a; Unterman *et al.*, 1993b; Unterman *et al.*, 1991), while cortisol administration to humans also increases IGFBP-1 (Conover *et al.*, 1993c). The promoter region of IGFBP-1 contains a glucocorticoid responsive region through which these effects may be mediated (Suwanichkul, *et al.*, 1993; Goswami *et al.*, 1994). Dexamethasone treatment of rats also stimulates circulating IGFBP-3 and hepatic IGFBP-3 mRNA (Luo *et al.*, 1990). In contrast to IGFBP-1 and IGFBP-3, Orlowski *et al.* (1990b) have shown a reduction in IGFBP-2 mRNA in the livers of young rats treated with glucocorticoids. Glucocorticoids inhibit IGFBP-3, -4 and -5 protein and mRNA levels, stimulate IGFBP-1 mRNA but have no effect on IGFBP-6 mRNA in cultured human osteoblast-like cells (Okazaki *et al.*, 1994). These observations demonstrate the contrasting regulation of different IGFBPs by glucocorticoids, in an IGFBP specific manner.

The IGFBPs are also regulated by many other factors such as retinoic acid, steroid hormones such as estrogen and progesterone, other IGFBPs and intracellularly by cAMP and protein kinase C (Rechler, 1993).

1.2.5.2 Regulation of IGFBPs by proteolysis

The levels of IGFBP and their IGF binding affinity can be regulated by specific proteolytic degradation or cleavage. Proteolytic activity for IGFBPs was first observed in maternal rat blood during pregnancy. During late pregnancy in the rat, maternal IGFBP-2, IGFBP-3 and IGFBP-4 are proteolytically degraded, resulting in an apparent decline in the levels of these IGFBPs, as measured by Western ligand blot (Davenport *et al.*, 1990; Gargosky *et al.*, 1990a; Rechler, 1993). Similar protease activity is present in human maternal blood throughout pregnancy (Hossenlopp *et al.*, 1990; Giudice *et al.*, 1990;

Gargosky *et al.*, 1991). In contrast to IGFBP-3 proteolytic activity in the rat, circulating maternal IGFBP-3 in the human is only partially proteolysed and retains the ability to form the 150 kDa complex, although with an apparently reduced affinity for IGF (Suikkari and Baxter, 1991; Davies *et al.*, 1991a). Protease activity for various IGFBPs have subsequently been reported in human plasma during post-operative recovery, long term illness such as cancer, and in fluids such as seminal plasma (Davies *et al.*, 1991b; Davenport *et al.*, 1992; Frost *et al.*, 1993). Cultured cells also produce IGFBP proteases (Fowlkes and Freemark, 1992a; Grimes and Hammond, 1994; Kanzaki *et al.*, 1994). Proteolytic activity for human IGFBP-3 from cultured bovine fibroblasts has been reported that promotes IGFBP-3 cell association and decreases IGF binding affinity (Conover, 1992). Proteases for IGFBP-3, IGFBP-4 and -5 have been described that are produced by a variety of cell types whose proteolytic activity can be promoted or inhibited by IGF in a manner independent of receptor binding and dependent on the ability of IGF to bind to IGFBPs (Fowlkes and Freemark, 1992a; Neely and Rosenfeld, 1992; Myers *et al.*, 1993; Kanzaki *et al.*, 1994). Thus, the IGFs may directly regulate IGFBP proteolysis by association with IGFBPs, or alternatively, but less likely, through interaction with the protease itself. Through these novel mechanisms, specific proteolytic cleavage or degradation of IGFBPs may regulate both the amount of IGFBP and its affinity for IGF, ultimately effecting the bio-availability of IGF at the cell surface.

1.2.5.3 Regulation of IGFBPs by post-translational modification

Several of the IGFBPs undergo post-translational protein modifications which offer another level of regulation of IGFBPs. Recent work has shown by two-dimensional (2-D) gel electrophoresis that there is large variability in IGFBP size and charge, suggesting wide spread post-translational modification of many IGFBPs (Chan and Nicoll, 1994). IGFBP-1, IGFBP-3, IGFBP-4 and IGFBP-6 have variable levels of N-linked, and in the case of IGFBP-6 O-linked, glycosylation, of unknown function. However, recombinant, non-glycosylated human IGFBP-3 has similar IGF binding properties to recombinant, glycosylated human IGFBP-3, suggesting that glycosylation of IGFBP-3 does not affect

IGF affinity (Sommer *et al.*, 1993). Glycosylation of recombinant human IGFBP-3 is also not required for IGFBP-3 potentiation of IGF stimulated DNA synthesis in fibroblasts (Conover *et al.*, 1991). Additionally, recombinant non-glycosylated human IGFBP-3 forms the 150 kDa complex, indicating that glycosylation is not essential for ternary complex formation (Sommer *et al.*, 1993). IGFBP-1 exists in several phosphorylated forms and the level of phosphorylation has been shown to increase the affinity of IGFBP-1 for IGFs, with highly phosphorylated IGFBP-1 having greatest IGF affinity (Jones *et al.*, 1991). The types of post-translational modification of IGFBPs that occur and their effects on IGFBP and IGF action have not been extensively studied and requires further investigation.

Thus, the actions of the IGFBPs, and ultimately that of the IGFs, may be altered through changes in circulating IGFBP levels, exerted through changes in mRNA levels or proteolytic degradation, or changes in IGFBP affinities for IGF, through proteolytic cleavage or post-translational modification of IGFBPs (Table 1.3).

1.3 FETAL GROWTH AND DEVELOPMENT

One of the most important determinants of fetal growth and development is the adequate supply of substrates, such as glucose, and oxygen to the growing fetus. The provision of these nutrients to the fetus is strongly influenced by placental transfer from the mother. Equally important is the ability of the fetus to utilise available substrates, which is primarily dependent on the actions of hormones and growth factors in the fetal circulation and within target tissues. Many endocrine and local factors are important for fetal growth and development. Epidermal Growth Factor (EGF) plays an important role in lung maturation in late gestation (Minoo and King, 1994). Although EGF mRNA cannot be detected in the rodent embryo or fetus, the EGF receptor is widely expressed throughout gestation, particularly in the placenta (Han and Hill, 1994). Several members of the Transforming Growth Factor-β (TGF-β) family are present in the fetus and embryo and are suggested to be involved in embryogenesis and specifically in bone growth (Heath and Smith, 1989; Whitman and Melton, 1989; Han and Hill, 1994). Platelet Derived Growth

Factor (PDGF) is expressed in the developing embryo and may have an important role, while the fibroblast growth factor family (FGF) are essential for mesoderm induction and appropriate development of the early xenopus embryo (Heath and Smith, 1989; Whitman and Melton, 1989). In post-natal life, growth is GH and IGF dependent. In contrast, even though the fetus contains high circulating GH levels, loss of the pituitary does not profoundly alter fetal growth which thus appears largely GH independent (Gluckman, 1986; Bassett *et al.*, 1989; Han and Hill, 1994). However, hypothyroid fetuses are growth retarded, indicating an important role of Thyroid Hormones in fetal growth and development (Gluckman, 1986; Han and Hill, 1994). The thyroid is known to influence and produce IGFs and IGFBPs (1.2.5.1) and thus growth retardation of the hypothyroid fetus may be related, in part, to alterations in fetal IGF and IGFBP levels. Although the complete endocrinology and expression of local factors required for normal fetal growth and development is complex, one element common to many perturbations of fetal growth are alterations in circulating insulin or the IGF / IGFBP system.

1.3.1 INSULIN IN FETAL GROWTH AND DEVELOPMENT

Insulin has been suggested for many years to be important for fetal growth. Insulin peptides and receptors are present from the early stages of embryogenesis, throughout gestation (De Pablo *et al.*, 1990). Insulin is active in the embryo, stimulating DNA and protein synthesis, while insulin or insulin-receptor antibodies retard embryonic growth in the chick (De Pablo *et al.*, 1990). Culture of rat embryos under conditions of low insulin bioactivity also leads to retardation of embryonic growth and development (Travers *et al.*, 1992). Alterations in fetal insulin levels positively correlate with changes in fetal growth (Gluckman, 1986; Fowden, 1989; Chard, 1989). Increased fetal growth is associated with high insulin levels and conversely, fetal growth retardation is associated with low fetal insulin in the sheep (Fowden, 1989; Fowden *et al.*, 1989; Phillips *et al.*, 1991a). Changes in insulin levels are often accompanied by alterations in levels of other factors, such as the IGFs. Thus, although insulin is clearly an important factor for fetal growth, it may act in conjunction with or be dependent on other important factors *in utero*, such as the IGFs.

1.3.2 THE IGFs IN FETAL GROWTH AND DEVELOPMENT

As in the adult, the IGFs are produced by most tissues and many cell types of the fetus and embryo (Han *et al.*, 1987). IGF-I mRNA is not present in the pre-implantation mouse embryo but becomes detectable in early post-implantation embryos (De Pablo *et al.*, 1990). IGF-II mRNA is present as early as the 2 cell stage of embryogenesis in the mouse (Heyner *et al.*, 1989). Both IGF-I and IGF-II mRNA are present in a tissue specific manner throughout early organogenesis in the rat (Bondy *et al.*, 1990), while IGF-I and IGF-II mRNAs are also abundant in the developing rat limb bud and their expression changes during limb outgrowth and differentiation, suggesting a role in this process (Streck *et al.*, 1992). In mid gestation human fetal tissues, IGF-I and IGF-II mRNAs are present at many sites such as muscle, liver and kidney, while hybridisation to IGF-II mRNAs was more intense than IGF-I in all tissues studied (Han *et al.*, 1987; Han and Hill, 1994). The type I IGF, insulin receptor and type II IGF / cation-independent mannose-6-phosphate receptor are also present in many tissues of the fetus and embryo and detectable from as early as the 8 cell stage of the pre-implantation embryo (Heyner *et al.*, 1989, De Pablo *et al.*, 1990). Thus, both IGF peptides and receptors are widely distributed throughout the fetus and embryo across an extensive time frame of development, suggesting the action of these growth factors throughout growth and development.

1.3.2.1 Developmental Regulation of IGFs

Although present throughout most of embryonic and fetal development, the IGFs are not constitutively expressed and are regulated in a tissue specific and developmental fashion (Cohick and Clemmons, 1993; Han and Hill, 1994). For example, circulating IGF-I levels are low at early gestational ages and increase with advancing fetal or neonatal age in the rat or sheep (D'Ercole *et. al.*, 1980; Moses *et al.*, 1980; Gluckman and Butler, 1983; Mesiano *et al.*, 1989). IGF-II is present in many fetal and embryonic structures but becomes restricted to specific tissues such as the liver, kidney and brain in the late gestation ovine or human fetus or the adult rat (Lund *et al.*, 1986; Han *et al.*, 1987; Delhanty and

Han, 1993). Many studies, including data presented in Chapter 5 (Carr *et al.*, 1994b), have shown that IGF-II levels are generally higher in the fetus than the adult, while the reverse is true for IGF-I, in species such as the human, sheep and rat (Moses *et al.*, 1980; Gluckman and Butler, 1983; Cohick and Clemmons, 1993; Han and Hill, 1994). In the most extreme case, circulating IGF-II is present in the fetal rat, but undetectable in the adult while IGF-I is undetectable in the fetus but abundant in the adult rat (Moses *et al.*, 1980; Han and Hill.,1994) (Table 1.4). Gene knock-out experiments have provided direct evidence for the importance of IGF-I and IGF-II in fetal growth and development. Knock-out of the mouse IGF-II gene, results in fetal growth retardation to approximately 60 % of normal body weight, although resultant mice are viable (De Chiara *et al.*, 1990) (Table 1.4). In contrast, gene knock-out of the type I IGF receptor is lethal in the homozygous mouse, due to respiratory failure, and is additionally characterised by growth retardation, muscle and general organ hypoplasia (Liu *et al.*, 1993a). Knock-out of the IGF-I gene has been performed by two independent investigators. Liu *et al.* (1993a) report homozygous IGF-I deficient mice to be growth retarded to a similar extent to the IGF-II gene knock-out mice and further report lethality in some of these animals (Table 1.4). Powell-Braxton *et al.* (1994) also show growth retardation in heterozygous IGF-I deficient mice, but report growth retardation, severe muscular dystrophy, an inability to breathe and greater than 95 % lethality in homozygous IGF-I deficient mice (Table 1.4). These results closely reflect the type I receptor homozygous knock-out mice of Liu *et al.* (1993a), and are suggestive of a particular role of IGF-I in muscle development. Thus, it is unquestionable that both IGF-I and IGF-II are essential for normal growth, development and viability of the fetus.

1.3.2.2 The IGF-II 'sink' hypothesis

IGF-II gene knock-out experiments indicated that heterozygous mice whose paternal IGF-II gene had been ablated, but not heterozygotes with the maternal IGF-II gene disrupted, were growth retarded (De Chiara *et al.*, 1990). This suggested maternal imprinting and silencing of the IGF-II gene in the mouse (De Chiara *et al.*, 1991). The type II IGF / cation-independent mannose-6-phosphate receptor is imprinted in a reprocial

	FETUS	ADULT	GENE KNOCK-OUT PHENOTYPE
IGF-I PROTEIN	+ (ND, rat)	+++	lethal (variable) growth retardation muscular dystrophy
IGF-I mRNA DISTRIBUTION AND ABUNDANCE	ubiquitous +	ubiquitous +++	
IGF-II PROTEIN	+++	+ (ND, rat)	60 % reduction in body weight
IGF-II mRNA DISTRIBUTION AND ABUNDANCE	ubiquitous +++	restricted (brain, liver, kidney, adrenal) ++	
IGFBP-3 PROTEIN	+ (ND, rat)	+++	not determined
IGFBP-3 mRNA DISTRIBUTION AND ABUNDANCE	ubiquitous +	ubiquitious +++	
IGFBP-2 PROTEIN	+++	+ (ND, rat)	no phenotypic effect
IGFBP-2 mRNA DISTRIBUTION AND ABUNDANCE	ubiquitious (early) restricted (mid- late) +++	restricted ++	

TABLE 1.4. SUMMARY OF IGFs AND IGFBPs IN THE FETUS AND THE ADULT. Alterations in circulating levels and mRNA distribution and abundance for IGF-I, IGF-II, IGFBP-2 and IGFBP-3, between the fetus and the adult are shown. The summary is generalised, as species specificity in absolute levels of these factors exist. Ubiquitous indicates the mRNA is present in many tissues, + indicates level of abundance, ND = not detectable in the rat circulation. Data is described fully and referenced in 1.3.2 and 1.3.3.

↓ Fetal weight and size	↓ Insulin
↓ Placental weight	↑ Cortisol
Organ assymetry (eg. ↑ brain : body weight ratio)	↑ Catecholamines
Altered morphology (eg ↓ placental development)	↓ IGF-I
↓ pO2	↑↓ IGF-II
↑ pCO2	
↓ Glucose	

TABLE 1.5 CHARACTERISTICS OF INTRA-UTERINE GROWTH RETARDATION INDUCED BY RESTRICTED PLACENTAL IMPLANTATION. Fetal growth retardation induced by surgical restriction of placental implantation is characterised by gross fetal and organ weight changes and morphological alterations, as well as endocrine perturbations (1.3.4), (Robinson *et al.*, 1994).

manner and only expressed from the maternal genome in the mouse (Barlow *et al.*, 1991). From these observations, the 'sink hypothesis' was derived to describe the role of the type II IGF receptor and IGF-II in the fetus (Haig and Graham, 1991). This hypothesis suggests that high levels of IGF-II *in utero* leads to large fetal size, while the role of the type II IGF receptor is to negate these effects of IGF-II and act as a 'sink' for inactivating circulating IGF-II and degrading IGF-II at the cell surface. The hypothesis also argues the reproductive logic of the reciprocal maternal / paternal imprinting of these two genes. It suggests that the male aims to produce the biggest and strongest offsrping (that are presumably more viable and dominant post-natally) by ensuring high levels of IGF-II and low levels of the type II IGF receptor in the fetus. Conversely, the female aims to produce a viable fetus with minimal compromise to her fitness or other offspring, by ensuring high amounts of the type II IGF receptor, to combat the paternally derived IGF-II, with little IGF-II expression from the maternally derived gene. Evidence to support this hypothesis has been derived from the evolutionary observation that in egg laying species, such as chickens, the cation-independent mannose-6-phosphate receptor does not bind IGF-II (Clairmont and Czech, 1989; Yang *et al.*, 1991). However, further investigations, in species other than the mouse, have shown that although human IGF-II is imprinted (Giannoukakis *et al.*, 1993), the type II IGF / cation-independent mannose-6-phosphate receptor is not (Kalscheuer *et al.*, 1993; Ogawa *et al.*, 1993a). Alterations in IGF-II imprinting and cellular release of IGF-II have been observed in tumour associated diseases such as Beckwith-Weideman Syndrome (Weksberg *et al.*, 1993) and Wilms Tumour (Ogawa *et al.*, 1993b), suggesting a functional role of IGF-II imprinting in regulation of IGF-II and control of cell growth. However, the role of imprinting in affecting the actions of IGF-II and the type II IGF receptor, and the implications of this for fetal growth and development, remain speculative.

1.3.3 THE IGFBPs IN FETAL GROWTH AND DEVELOPMENT

Like the IGFs, the IGFBPs are present in many tissues in the fetus and embryo from very early embryogenesis and throughout the gestational period. IGFBP-2, -3 and -4 mRNAs have been shown to be present at all stages of the pre-implantation embryo and IGFBP-6 mRNA is detectable in the blastocyst (Hahnel and Schultz, 1994). IGFBP-5 mRNA is not detectable at any stage of the pre-implantation embryo, but is expressed in the early post-implantation mouse embryo, with a pattern of cellular localisation distinct from that of IGFBP-2, and showing developmental regulation (Hahnel and Schultz, 1994; Green *et al.*, 1994). Several studies have investigated the localisation of IGFBPs 1 to 6 in the late gestation human fetus and mid to late gestation rat and mouse fetus, and indicate localisation of different IGFBPs to distinct cellular sites (Delhanty *et al.*, 1993; Schuller *et al.*, 1993; Cerro *et al.*, 1993). IGFBP-2 mRNA is present at early embryonic and later stages in the rodent and is localised to regions complementary to that of IGF-II mRNA (Wood *et al.*, 1992). IGFBP-2 mRNA is present in many tissues of the fetus but becomes restricted to the liver, kidney and choroid plexus with advancing gestational age in the sheep, human or rat, in a manner similar to that for IGF-II (Delhanty and Han, 1993; Han and Hill, 1994). Thus, the IGFBPs are present throughout gestation and show specific localisation and expression patterns, suggesting specific functions for IGFBPs in growth and development of particular tissues.

1.3.3.1 Developmental Regulation of IGFBPs

The IGFs are important factors for fetal growth and development (1.3.2) and thus the IGFBPs (which are present throughout gestation and modulate the action of the IGFs) can also be considered an important family of proteins for modulating fetal growth and development

Several studies have suggested developmental regulation of IGFBPs, due to differences in the circulating IGFBP profiles between the adult, fetus and neonate (Butler and Gluckman, 1986; Donovan *et al.*, 1989; Liu *et al.*, 1991; Wang and Chard, 1992; Lee *et al.*, 1993a) (Table 1.4) and developmental differences in IGFBPs produced by cells of pre-

or post-natal origin (Fowlkes and Freemark, 1992b). Studies in the sheep have described developmental changes in IGFBP-1 and IGFBP-2 mRNA levels in several tissues throughout gestation (Phillips *et al.*, 1991b; Delhanty and Han, 1993) although the developmental regulation of other more recently characterised IGFBPs, such as IGFBP-4, has not been investigated. In general, IGFBP-2 is higher in the fetus than the adult, while the reverse is true of IGFBP-3 in the early to mid gestation fetus compared to the adult (Liu *et al.*, 1991; McCusker *et al.*, 1991; Lee *et al.*, 1993a) (Table 1.4). The rat again represents the most extreme case with high circulating IGFBP-2 levels in the fetus but undetectable levels in the adult, and undetectable circulating IGFBP-3 in the rat fetus, but abundant IGFBP-3 in the adult (Donovan *et al.*, 1989) (Table 1.4). This situation is the same as that described in 1.3.2.1 for IGF-II. Therefore, we can present an analogous argument suggesting a specific role for IGFBP-2 in the fetus. However, in contrast to the IGF-II gene knock-out, recent gene knock-out experiments of IGFBP-2 in the mouse have failed to show any major phenotypic effect on fetal growth and development (Pintar *et al.*, 1994) (Table 1.4). These observations may indicate redundancy in IGFBP function with the lack of IGFBP-2 being accommodated for by other IGFBPs. This functional redundancy of IGFBPs suggests that further investigation of other IGFBPs in fetal growth and development is required to fully understand the complex interactions within the IGFBP system that may be involved in regulating fetal growth and development.

1.3.4 INTRA-UTERINE GROWTH RETARDATION

Intra-uterine growth retardation (IUGR) is a major problem in both clinical medicine and animal production with increased risks of pre- and post-natal mortality and susceptibility to adult disease associated with reduced weight at birth (Alexander, 1974; Barker, 1992). Intra-uterine growth retardation can result from many different situations such as stress, undernutrition and multiple pregnancies. Experimental restriction of growth *in utero* can be induced by techniques such as a reduction of uterine blood flow, maternal fasting, glucocorticoid administration, heat stress or restriction of placental implantation (Alexander, 1974; Owens *et al.*, 1989). When placental substrate supply is limited by

restriction of placental implantation in the sheep, the feto-placental weight ratio is increased, although total fetal body weight is decreased (Robinson *et al.*, 1979; Robinson *et al.*, 1994). The organ to body weight ratios of tissues such as the brain are increased, while tissues such as the liver, lung, heart and spleen are reduced in proportion to body weight, indicating asymmetric growth and sparing of some essential tissues (Robinson *et al.*, 1979; Robinson *et al.*, 1994). In addition to changes in organ weights the morphology of some organs and tissues, such as the brain, placenta, gastrointestinal tract and lung, may be altered (Rees, *et al.*, 1991; Trahair *et al.*, 1993; Robinson *et al.*, 1994). Wool development is also severely affected in the mid-gestation growth retarded fetal sheep (Harding *et al.*, 1985) In severe intra-uterine growth retardation, the blood gas content may change, with fetuses becoming hypoxic and hypercapnic (Harding *et al.*, 1985; Owens *et al.*, 1994). Glucose and insulin levels are decreased while cortisol levels are increased in sheep fetuses subjected to restricted placental implantation (Robinson *et al.*, 1979; Harding *et al.*, 1985; Owens *et al.*, 1989). Circulating IGF-I is decreased in the growth restricted ovine fetus while circulating IGF-II has been reported to both increase (Jones *et al.*, 1988) or decrease (Owens *et al.*, 1994). This apparent discrepancy in changes in circulating IGF-II may be due to a superimposed effect of gestational age on restriction, with circulating IGF-II levels being initially elevated, then decreased in late gestation in the restricted ovine fetus. Alternatively, or additionally, apparent increases in circulating IGF-II may be due to inaccurate measurements of IGF-II levels due to the interference of IGFBPs not adequately removed in some studies. The characteristics of IUGR induced by experimental restriction of placental implantation in the sheep are summarised in Table 1.5. IGFs are also altered in other models of growth restriction. Growth retardation, induced by uterine artery ligation in the late gestation rat, results in decreases in circulating somatomedin or IGF-I, glucose and insulin levels, but has no effect on circulating IGF-II levels (Vileisis and D' Ercole, 1986; Unterman *et al.*, 1993a). Growth retardation induced by maternal fasting in rats also results in lower IGF-I levels (Bernstein *et al.*, 1991), while a decline in circulating and liver IGF-II mRNA with growth retardation induced by maternal fasting has also been observed in the rat (Straus *et al.*, 1991; Donovan *et al.*, 1991). Fetal growth retardation in the rat, induced

by maternal hypoxia, is associated with low circulating IGF-I and elevated circulating IGF-II levels (Tapanainen *et al.*, 1994).

The IGFBPs have been investigated in acute experimental models of fetal growth retardation induced by dexamethasone treatment, uterine artery ligation, maternal fasting or maternal hypoxia, in the rodent (Price *et al.*, 1992; Donovan *et al.*, 1991; Straus *et al.*, 1991; Unterman *et al.*, 1993a and 1993b; Tapanainen *et al.*, 1994). In the small-for-gestational-age rat, induced by uterine artery ligation, circulating IGFBP-1, but not IGFBP-2 is increased (Unterman *et al.*, 1990; Unterman *et al.*, 1993a), while growth retardation in rats caused by maternal fasting is characterised by increased hepatic IGFBP-1, but not IGFBP-2 mRNA. In contrast, in the neonatal rat where food intake of the mother is restricted, circulating IGFBP-2 is increased (Donovan *et al.*, 1991) while in growth retardation induced by maternal hypoxia, circulating IGFBP-1, IGFBP-2 and IGFBP-4 are increased (Tapanainen *et al.*, 1994). Dexamethasone induced fetal growth retardation increased circulating IGFBP-1, but not IGFBP-2, although IGFBP-2 liver mRNA was elevated (Price *et al.*, 1992; Unterman *et al.*, 1993b). Therefore, the common response of the IGF / IGFBP axis to growth retardation seems to be decreased circulating IGF-I and hepatic IGF-I mRNA, increased circulating IGFBP-1 and hepatic IGFBP-1 mRNA, with variable increases or decreases in IGF-II and IGFBP-2.

Insulin, IGFs and IGFBPs have all been implicated as important determinants of fetal growth (1.3.1 to 1.3.3) and thus, fetal growth retardation may result from a complex interaction of factors, involving changes in many important mediators of growth.

1.4 AIMS AND SIGNIFICANCE OF THIS THESIS

The aims of these studies were to investigate the roles of the IGFBPs in fetal growth and development. In particular, it was hypothesised that:

- The IGFBPs regulate growth and development of the ovine fetus.
- The IGFBPs are developmentally regulated throughout gestation, both in the circulating protein and the mRNA levels, in a tissue specific manner in the ovine fetus.

- IGFBP-4 is present in the ovine fetus and is developmentally regulated in a tissue specific manner.
- The developmental changes in IGFBPs relate to circulating IGF levels.
- The IGFBPs partly mediate the restriction of fetal growth when placental growth and function is limited.

These hypotheses were addressed through an analysis of the ontogeny of IGFBPs, including IGFBP-4, in the ovine fetus throughout normal development and following placental restriction of substrate supply to the fetus. More specifically this study has:

- Isolated and characterised the cDNA sequence of ovine IGFBP-4.
- Isolated and characterised a specific ovine IGFBP-4 antibody.
- Characterised circulating IGFBP-2, IGFBP-3 and IGFBP-4, and IGFBP-2 and IGFBP-4 mRNAs in major fetal tissues throughout gestation in the ovine fetus.
- Characterised the relationship between circulating IGF-I and IGF-II and IGFBPs in the ovine fetus.
- Characterised the effect of chronic placental restriction of fetal growth on the ontogeny of the IGFBPs axes in the ovine fetus.

At the time of commencement of this project, little was known of IGFBP-4 or the ontogeny of IGFBPs in any species, other than the rat. Furthermore, the developmental changes in IGFBP mRNA in specific tissues and of IGFBPs, in a chronic model of *in utero* growth restriction in a large mammalian species, had not been extensively addressed. Thus, the studies in this thesis aimed to provide novel information on the recently isolated IGFBP-4, in addition to further information on other circulating IGFBP and IGFBP tissue mRNA levels, over an extended period of gestation in normal and perturbed fetal growth, in a higher mammalian species.

Although such descriptive data does not directly address the roles of the IGFBPs, results from these studies are an essential pre-requisite to identify organ system and developmental stages where IGFBPs may be particularly important (eg. IGFBP-2 or

IGFBP-4 in early heart or lung development). Correlations of observed changes in IGFBPs with other endocrine factors has also identified potential regulators of IGFBPs *in utero*, which can be further investigated (eg. IGFBP-2 and cortisol). Additionally, data described in this thesis has extended our understanding of the endocrinology of the ovine fetus, in normal and growth restricted pregnancies.

CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

NaCl, Na-acetate (NaOAc), NH_4OAc, bromophenol blue, xylene cyanol, trichloro acetic acid (TCA), boric acid, glucose, Triton X-100, chloroform and acetic acid were obtained from BDH Chemicals, Australia, Pty. Ltd., Kilsyth, Vic., Australia. Sodium citrate, $MgCl_2$, $CaCl_2$, NaOH and polyethylene glycol (PEG) 6000 were obtained from AJAX Chemicals, Sydney, NSW, Australia. $NaH_2PO_4.2H_2O$, NH_4Cl, LiCl and KH_2PO_4 were obtained from May and Baker Australia Ltd., West Footscray, Vic., Australia. Ethylenediaminetetraacetic acid (EDTA) was from Boehringer Mannheim, Germany. Ficoll 400, Sephadex G-50 and Sepharose CL-6B were obtained from Pharmacia, Uppsala, Sweden. Tris-(hydroxymethyl)methylamine (Tris), acrylamide and guanidinium thiocyanate were from Merk Pty. Ltd., Kilsyth, Vic., Australia. Bis-acrylamide was obtained from Biorad Laboratories Inc., Herates, CA, USA. Sucrose was obtained from CSR Ltd. Refined Sugars, North Sydney, NSW, Australia. Phenol (special grade) was from Wako Pure Chemical Industries Ltd., Osaka, Japan. Dimethyl formamide (DMF) was obtained from Mallinckrodt Chemical Works, St Louis, MI, USA. $Na^{125}I$ was from Amersham Australia, North Ryde, NSW, Australia. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Diagnostic Chemicals Ltd., Charlottown, Canada. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG, X-GAL) was obtained from Progen Industries Ltd. Darra, Qld., Australia and GENECLEAN II kit was purchased from Bio101 Inc., La Jolla, CA, USA. Bactotryptone, bactoyeast extract and bactoagar were all obtained from DIFCO Laboratories, MI, USA. Isopropanol (propan-2-ol) was obtained from FSE, Homebush, NSW, Australia. Polyoxyethylene-sorbitan monolaurate (Tween-20), polyvinylpyrrolidone (PVP), agarose, *E. coli* transfer RNA (tRNA, type XX), ampicillin, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), N-lauroylsarcosine (sarkosyl), sodium dodecyl sulphate (SDS), 8-hydroxyquinoline, β-mercaptoethanol (2-

hydroxyethylmercaptan), dithiothreitol (DTT; molecular biology reagent), dithioerythritol (DTE), spermidine (N-[3-aminopropyl]-1,4-butanediamine), salmon testis (sperm) DNA and glass beads (212 to 300 µm) were all obtained from Sigma Chemical Co. St. Louis, MO, USA. High Efficiency Transfer System (HET-S) was obtained from Cinna/Biotex Laboratories Inc., Houston, Texas, USA. Nitrocellulose and Nytran membranes (0.45 µm pore size) were obtained from Schleicher and Schuell, Dassel, Germany. Whatman 3MM paper was obtained from Whatman International Ltd., Maidstone, England and x-ray film was obtained from Konica Corporation, Tokyo, Japan.

2.1.2 PROTEINS, PEPTIDES AND ENZYMES

Gigaprime DNA labelling kit, [α-^{32}P]dATP, [γ-^{32}P]rATP, [α-^{35}S]dATP, T4 polynucleotide kinase (PNK) and T4 DNA ligase were obtained from Bresatec, Ltd., Thebarton, South Australia. Calf intestinal alkaline phosphatase (CIP) was from Boehringer Mannheim, Germany. Sequenase v2.0 and deoxyinosine triphosphate (dITP) sequencing mix were obtained from United States Biochemical (USB), Cleveland, OH, USA. Restriction endonucleases were obtained from Pharmacia, Uppsala, Sweden. Deoxynucleotide triphosphate and dideoxynucleotide triphosphate mixes (dNTP and ddNTP, respectively) were obtained from Biotech International, Bentley, Western Australia. DNase free RNase A, adenosine 5'-triphosphate ribonucleoside (rATP), bovine serum albumin (BSA), avidin alkaline phosphatase-biotin conjugate and goat anti-rabbit IgG-biotin conjugate were all obtained from Sigma Chemical Co. St. Louis, MO, USA. Recombinant human IGF-I was a gift from Genentech Inc. San Francisco, CA. USA, and recombinant human IGF-II was obtained from GroPep Pty. Ltd., Adelaide, South Australia. ^{14}C-labelled molecular weight rainbow markers were obtained from Amersham Australia, North Ryde, NSW, Australia. A 0.24 to 9.5 kb RNA ladder was purchased from Gibco BRL, Gaithersburg, MD, USA. Sequencing primers were obtained from Bresatec, Ltd., Thebarton, South Australia.

Reverse sequencing primer (RSP) 5'CACACAGGAAACAGCTATGACCATG 3'

Universal sequencing primer (USP) 5' GTAAAACGACGGCCAGT 3'

2.1.3 SOLUTIONS AND BUFFERS

LB (Luria-Bertani medium)

1 % (w/v) bactotryptone

0.5 % (w/v) bactoyeast extract

0.17 M NaCl pH 7.0

LB + agar plates

LB + 1.5 % (w/v) bacto-agar

LB + agarose plates

LB + 0.7 % (w/v) agarose

2YT

1.6 % (w/v) bactotryptone

1 % (w/v) bactoyeast extract

0.085 M NaCl pH 7.0

Min A media

0.05 M Na_2HPO_4

0.022 M KH_2PO_4

8 mM NaCl

0.02 M NH_4Cl

0.4 % (w/v) glucose

50 X Denharts

1 % (w/v) Ficoll 400

1 % (w/v) Polyvinylpyrrolidone (PVP)

1 % (w/v) BSA

20 X SSPE

3 M NaCl

0.2 M $NaH_2PO_4.H_2O$

0.02 M EDTA pH 7.4

20 X SSC

3 M NaCl

0.3 M sodium citrate pH 7.0

20 X TBE

1 M Tris pH 8.3

0.02 M EDTA

0.8 M boric acid

10 X Superduper Buffer

0.33 M Tris-OAc pH 7.8

0.625 M KOAc

0.1 M MgOAc

0.04 M spermidine

0.005 M DTE

1 X TE

10 mM Tris pH 7.5

0.1 mM EDTA

10 X PNK

0.25 M Tris pH 9.0

0.05 M $MgCl_2$

0.05 % (w/v) BSA

0.01 M DTT

0.01 M rATP

10 X Ligase Buffer

0.5 M Tris pH 7.5

0.1 M $MgCl_2$

0.01 M DTT

0.01 M rATP

40 % (w/v) PEG 6000

10 X CIP

0.5 M Tris pH 7.5

0.1 M $MgCl_2$

E. coli Strains

DH5α : *supE*44Δ*lacU*169(ø80*lacZ*ΔM15)*hsdR*17*recA*1*endA*1*gyrA*96*thi*-1*relA*1

MV1190 : Δ(*lac-proAB*)*thisupE*Δ(*sr1-recA*)306::Tn10(*tet*^{r})

F'[*traD*36*proAB*$^+$*lacI*^{q}*lacZ*ΔM15]

(Sambrook *et al.*, 1989)

Preparation of Phenol

Crystallised phenol was melted at 68° C and 0.1 % (w/v) 8-hydroxyquinoline added. Phenol was Tris-saturated by extraction with 1 M Tris, pH 8.0 followed by 0.1 M Tris pH 8.0, several times, until the pH of the aqueous phase was greater than 7.6. Water-saturated phenol was prepared by melting crystalline phenol, as above and extracting with several volumes of water until the pH of the aqueous phase was approximately 7.0.

2.2 METHODS

2.2.1 DNA TECHNIQUES

2.2.1.1 Growth and transformation of E. coli

Glycerol stocks of *E. coli* strains MV1190 or DH5α were streaked out, aseptically, onto LB agar plates and grown overnight at 37° C. A single colony was inoculated into 5

ml LB and grown overnight at 37° C. Overnight cultures were subcultured, at 1/100 dilution, into LB and grown to A_{600} nm of 0.4 to 0.6. Cells were pelleted at 3,000 x g, five minutes at 4° C and gently resuspended in 1/20th the original volume of 50 mM $CaCl_2$, 20 mM $MgCl_2$. Cells were left on ice for at least one hour or overnight at 4° C to become competent. Circular DNA was incubated with 200 µl of competent cells on ice for 30 minutes then heat shocked by incubation at 42° C, two minutes then ice, one minute. An equal volume of LB was added and the cells incubated at 37° C for 10 minutes before plating.

2.2.1.2 Cloning DNA

Preparation of vector DNA

Plasmid DNA was digested with selected restriction enzymes under the appropriate salt and temperature conditions, as recommended by the enzyme manufacturer. The linearised vector was purified by GENECLEAN (2.2.1.4). Singly cut plasmid was dephosphorylated with 1 U of calf intestinal phosphatase (CIP) in 1 X CIP buffer at 37° C for 60 minutes. CIP was then either heat inactivated or removed on a Sepharose CL-6B spin column (2.2.1.4) before ligation.

Phosphorylation of DNA 5' ends

DNA (oligomers or PCR products) were heat denatured then cooled immediately on ice. 5' ends were phosphorylated with 3 U T4 polynucleotide kinase in 1 X PNK buffer at 37° C for 60 minutes. Kinase activity was inactivated at 65° C for 15 minutes or removed on a Sepharose CL-6B spin column (2.2.1.4). For radiolabelling of 5' ends, approximately 200 ng single stranded DNA was incubated, as above with the exception that 5 µl (50 µCi) [γ-^{32}P]rATP was substituted for 10 mM rATP.

Ligation of DNA

DNA inserts (5' phosphorylated) and linearised plasmids (dephosphorylated) were mixed in a ratio of approximately 2 : 1 and ligated in 1 X Ligase buffer with 2 U of T4 DNA

ligase overnight at 14° C. Negative controls (no DNA insert, vector alone) were always performed. DNA was transformed into *E. coli*, (2.2.1.1), and transformants with DNA inserts identified by plating with 500 µg IPTG and 1.2 mg BCIG and subsequent blue / white selection due to α-complementation of the β-galactosidase (*lacZ*) gene (Sambrook *et al.*, 1989). These substrates are metabolised to a blue precipitate in the presence of a functional *lacZ* gene product. Disruption the plasmid encoded portion of the *lacZ* gene by a DNA insert results in loss of functional β-galactosidase activity and a white colony when plated in the presence of IPTG and BCIG.

2.2.1.3 Preparation of plasmid DNA

Alkaline lysis method (miniprep; 2 ml culture)

Cells from a single colony were used to inoculate 2 ml LB + antibiotic (ampicillin at 50 µg/ml) and grown overnight at 37° C with shaking. Overnight culture (1.5 ml) was transferred to 1.5 ml tubes and the cells pelleted at 2,000 x g for five minutes. The supernatant was aspirated and pellets resuspended in 100 µl TES (25 mM Tris, pH 8.0, 10 mM EDTA, 15 % [w/v] sucrose). Freshly made 0.2 M NaOH, 1% (w/v) SDS (200 µl) and 3 M NaOAc, pH 4.6 (135 µl) were added and gently mixed by inversion. Genomic DNA was precipitated on ice for five minutes and pelleted by centrifugation for 15 minutes at 4° C, 8,000 x g. The supernatant was taken and digested with DNase-free RNase A (25 µg/ml) at 37° C for 30 minutes then extracted with an equal volume of 50 % (v/v) Tris-saturated phenol, 50 % (v/v) chloroform mix. The aqueous phase was recovered and precipitated with two volumes of ethanol at room temperature for 30 minutes. Precipitated DNA was pelleted by centrifugation, 8,000 x g, 4° C, for 15 minutes, washed with 70 % (v/v) ethanol and resuspended in 50 µl of 0.1 mM EDTA, pH 8.0. Approximately 1 µl of DNA was checked by agarose mini-gel electrophoresis for estimation of DNA quantity and purity.

2.2.1.4 DNA Purification

Sepharose CL-6B spin columns

Sepharose CL-6B spin mini-columns were used to desalt or purify DNA prior to sequencing or in between reactions. Two tubes (1.5 ml and 0.5 ml) were pierced with a 21 gauge needle. A mini-column was constructed in the 0.5 ml tube with a drop of glass beads (pre-washed in HNO_3 and rinsed in water) onto which approximately 0.5 ml of Sepharose CL-6B slurry in TE was overlaid. The 0.5 ml tube was placed inside the 1.5 ml pierced tube and then placed inside a 10 ml tube to collect the eluate. The mini-column was spun in a bench top centrifuge at approximately 1.8 rpm (200 to 500 x g) for three minutes. The pierced tube was replaced with a fresh intact 1.5 ml tube to recover the column eluate. The DNA sample (20 to 50 µl) was loaded onto the dry Sepharose CL-6B bed and respun, as above. An equivalent load volume was recovered in the 1.5 ml tube and contained DNA free of small nucleic acids, protein and salts. Recovery of DNA was checked by agarose mini-gel electrophoresis.

GENECLEAN isolation of DNA

DNA fragments, larger than approximately 400 bp, were purified using the GENECLEAN II kit, according to the manufacturers instructions. Briefly, DNA was electrophoresed on an agarose TBE gel, visualised by ethidium bromide staining and excised under long wave ultra violet (UV) light. The gel slice was solubilised in three volumes of NaI solution with 1/10th volume of TBE modifier and heated at 65° C for five minutes. Glassmilk solution (5 µl) was added and the DNA allowed to bind at room temperature for five minutes. Glassmilk beads were pelleted by centrifugation for 15 seconds at 10,000 x g and washed three times with New Wash solution, gently resuspending and pelleting, as above, in between washes. DNA was eluted from the glass beads with 10 µl of water by incubating at 65° C for five minutes. The glass beads were pelleted, as above, and the supernatant retained. The elution was repeated and supernatants combined. DNA recovery was checked by agarose mini-gel electrophoresis.

2.2.1.5 DNA Sequencing

DNA sequencing was performed, based on the dideoxy chain termination reactions of Sanger *et al.* (1977), with Sequenase v2.0, modified T7 DNA polymerase according to the manufacturers instructions. Briefly, DNA was denatured in 0.2 M NaOH, 0.2 mM EDTA at 37° C, 15 minutes, then desalted on a Sepharose CL-6B spin column (2.2.1.4). Denatured DNA (1 to 2 µg) and primer (RSP, USP or DNA specific primer, 10 to 50 ng) were mixed in a volume of 8 µl and 2 µl of annealing buffer added (200 mM Tris, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The reaction was heated at 65° C for three minutes then cooled slowly to room temperature, to denature the DNA then anneal the primer. The annealed primer was extended for three to five minutes at room temperature with 1 µl 0.1 M DTT, 2 µl labelling nucleotide mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 5 µCi [α-^{32}P]dATP or 10 µCi [α-^{35}S]dATP with 1 U of Sequenase enzyme in a final reaction volume of 15 µl. The extension reaction was terminated by adding 3.5 µl labelling reaction to 2.5 µl of each of the four ddNTP mixes (80 µM dATP, 80 µM dTTP, 80 µM dCTP, 80 µM dGTP, 50 mM NaCl with 8 µM ddATP, ddGTP, ddCTP or ddTTP) and incubating at 37° C for five minutes. The reactions were stopped by the addition of 4 µl of stop solution (95 % [v/v] deionised formamide, 20 mM EDTA, 0.05 % [w/v] bromophenol blue, 0.05 % [w/v] xylene cyanol) on ice. Sequencing compressions were resolved by substitution of dITP or ddITP for dGTP or ddGTP in the labelling and termination reactions, respectively. Sequencing reactions were denatured at 100° C for five minutes and loaded onto a 0.3 mm, 7 M Urea, 1 x TBE, 6 % polyacrylamide gel (w/v, 25 : 1, acrylamide : bis-acrylamide) and run at 40 Watts constant power (approximately 20 mA, 1500 V) for the appropriate migration distance. Gel plates were separated and the gel fixed by soaking for 30 minutes in 20 % (v/v) ethanol, 10 % (v/v) acetic acid, transferred to Whatman 3 MM paper and dried under vacuum at 65° C for 60 minutes. Dried gels were exposed to x-ray film at room temperature overnight (^{32}P) or for two to four days (^{35}S).

2.2.1.6 Generation of DNA probes

DNA probes were generated by the random priming method, as described by Feinberg and Vogelstein (1983), using the commercially available GIGAprime DNA labelling kit (Bresatec Ltd. Thebarton, South Australia). Briefly, double stranded DNA (approximately 200 ng) was heat denatured, annealed with random decanucleotide primers and extended with the Klenow fragment of *E. coli* DNA polymerase I in the presence of [α-^{32}P]-dATP at 37° C for 30 to 60 minutes. Labelled DNA was ethanol precipitated with 1/2 the volume of 7.5 M NH_4OAc in the presence of 25 µg *E. coli* tRNA, resuspended in 200 µl of 0.1 mM EDTA and 2 µl counted (LKB Wallac 1214 Rackbeta Liquid Scintillation Counter, LKB, Turku, Finland). Assuming the pelleted counts represented only incorporated [α-^{32}P]-dATP, specific activities of approximately 3 to 4 x 10^8 cpm/µg DNA were obtained.

2.2.2 RNA TECHNIQUES

2.2.2.1 RNA extraction

Total RNA was extracted according to the method of Chomczynski and Sacchi (1987), with slight modification. Briefly, approximately 1 g of frozen tissue was homogenised in 10 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5 % [w/v] sarkosyl) + 0.1 M β-mercaptoethanol. One tenth the volume of 2 M NaOAc, pH 4.0, an equal volume of water saturated phenol and 1/5th the volume of chloroform were added. The homogenate was mixed thoroughly and phases separated by centrifugation at 12,000 x g, 4° C for 20 minutes. The aqueous phase was recovered and precipitated with an equal volume of isopropanol at -20° C for one hour. The precipitate was pelleted by centrifugation at 8,000 x g, 4° C, 30 minutes, resuspended in 1/2 the original volume of TE + 0.1 % (w/v) SDS and re-precipitated with isopropanol, as above. The pellet was resuspended in 1/4 the original volume of TE + 0.1 % (w/v) SDS and precipitated overnight on ice with four volumes of 4 M LiCl. The pellet was recovered by centrifugation, 12,000 x g, 4° C, 30 minutes, resuspended and precipitated with 1/10th volume of 3 M NaOAc, pH 5.2 and two volumes of ethanol at -20° C, twice, centrifuging

as above, to recover the precipitated nucleic acids. Finally, the pellet was washed with 70 % (v/v) ethanol, air dried and resuspended in 0.5 to 1 ml of TE + 0.1 % (w/v) SDS. Purity, quantity and integrity of isolated RNA was assessed by agarose mini-gel electrophoresis and spectral analysis (2.2.4).

2.2.2.2 Northern analysis

RNA (50 µg) was electrophoresed on a 1 M formaldehyde, 10 mM sodium phosphate, 1 % agarose gel at 60 V for five hours. Separated RNA was transferred overnight to Nytran membranes by capillary action in HET-S (High Efficiency Transfer System). RNA was cross-linked to the membrane with UV light (UV Strata linker 1800, Stratagene, La Jolla, CA, USA) and intact RNA assessed by visualisation under UV light. Membranes were prehybridised (50 % deionised formamide, 5 X SSPE, 5 X Denharts solution, 0.1 % SDS, 0.05 % sodium pyrophosphate, 0.5 mg heat denatured, sonicated salmon sperm DNA) overnight at 42° C. DNA probes were generated by random priming, as described above. The probe (approximately 0.5 x 10^6 cpm/ml) was heat denatured, added to the prehybridisation mix and hybridised overnight at 42° C. Hybridised filters were washed sequentially at 42, 55 and 68° C in 2 X SSC, 0.1 % (w/v) SDS, then finally at 68° C in 0.1 X SSC, 0.1 % (w/v) SDS. RNA loading was assessed by reprobing the membrane with a rat 18S rRNA genomic clone (Katz *et al.*, 1983) as above, with the exception that hybridisations were performed at 55° C. Filters were exposed to x-ray film with intensifying screens at -80° C for varied time periods, as specified within each chapter. RNA transcripts were sized by co-electrophoresis of an RNA ladder. The RNA ladder was then visualised by probing the membrane with λ DNA and mRNA transcript size determined by construction of a standard curve of distance migrated from origin (data not shown).

2.2.3 PROTEIN TECHNIQUES

2.2.3.1 Western ligand blot

Plasma samples (20 µl of a 1 in 10 dilution, 50 mM phosphate, 0.15 M NaCl, pH 7.5 [phosphate-buffered saline, PBS]) were subjected to discontinuous non-reducing SDS-PAGE on a 4 % stacking gel and 12.5 % polyacrylamide separating gel (w/v, 40 : 1, acrylamide : bis-acrylamide) overnight at 20 mA (Laemmli, 1970). Separated proteins were transferred onto Nitrocellulose filters at 0.8 mA/cm^2 for one hour with a semi-dry electroblotter (Multiphor II Novablot, Pharmacia, Uppsala, Sweden) in 20 mM Tris, pH 8.3, 150 mM glycine, 20 % (v/v) ethanol. Membranes were wet with 10 mM Tris, pH 7.5, 150 mM NaCl (Tris-saline) + 1 % (v/v) Triton X-100 for 30 minutes, blocked with Tris-saline + 1 % (w/v) BSA for two hours then Tris-saline + 0.1 % (v/v) Tween-20 for 15 minutes and probed with ^{125}I-IGF-II (approximately 500,000 cpm) in 20 ml Tris-saline + 1 % (w/v) BSA, 0.1 % (v/v) Tween-20 overnight at 4° C, as described by Hossenlopp *et al.* (1986). Filters were washed in Tris-saline + 0.1 % (v/v) Tween-20 at 4° C for three hours, with buffer changes every 30 minutes, and exposed to x-ray film at -80° C with intensifying screens for two to three weeks. Molecular weights of IGFBP bands were calculated from a standard curve of migration distances of molecular weight markers (data not shown).

2.2.3.2 Western immunoblot

Proteins were electrophoresed and transferred to Nitrocellulose filters as described above for Western ligand blotting. Filters were blocked in Tris-saline + 1 % (w/v) BSA overnight at 4° C, rinsed in Tris-saline + 0.1 % (v/v) Tween-20 prior to overnight incubation at 4° C with the primary antibody, diluted in Tris-saline + 0.1% (v/v) Tween-20. Unbound antibody was removed by washing (three 20 minute buffer changes) in Tris-saline + 0.1% (v/v) Tween-20 at room temperature. The secondary antibody was added (anti-rabbit IgG-biotin conjugate at 1/20,000 in Tris-saline + 0.1% [v/v] Tween-20) and filters incubated for three hours at room temperature. Unbound secondary antibody was removed by washing, as above, followed by incubation with 0.05 µg/ml avidin alkaline phosphatase conjugate in Tris-saline + 0.1% (v/v) Tween-20 at room temperature for one hour. The

filters were washed for 20 minutes in Tris-saline + 0.1% Tween-20 then two 20 minute buffer changes in 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. The filters were developed by addition of BCIP and NBT to final concentrations of 0.2 and 0.3 mg/ml, respectively. The reaction was allowed to proceed until the appropriate colour intensity was achieved (approximately 15 minutes) and then stopped by the addition of EDTA at a final concentration of 1 mM.

2.2.3.3 Iodination of IGF

IGF-I and IGF-II peptides (10 µg) were iodinated to specific activities between 60 and 80 Ci/g with 1 mCi $Na^{125}I$ and 4 µg chloramine T, in a total volume of 130 µl for one minute. The reaction was stopped by the addition of 12 µg sodium metabisulphite (final volume, 150 µl). Radiolabelled IGF was purified on a Sephadex G-50, 1 x 50 cm column, in 50 mM sodium phosphate, pH 6.5, 150 mM NaCl + 0.25 % (w/v) BSA. Purified peptides were assayed for precipitability with 10 % (w/v) TCA and biological activity as described by Ballard *et al.*, 1986. All iodinations were performed by Mr. S. E. Knowles, CRC for Tissue Growth and Repair, Adelaide, South Australia.

2.2.4 SPECTRAL ANALYSIS

Concentration and purity of RNA, DNA or protein preparations was determined by spectral analysis using a Hewlett Packard, 8451A Diode Array Spectrophotometer. For RNA and DNA preparations the absorbance profiles $A_{210-320}$ were determined. The ratio A_{260}/A_{280} nm was approximately 1.6 to 1.7 (DNA) and 1.7 to 2.0 (RNA). Concentrations of protein, RNA and DNA solutions were calculated on the basis that a 1 mg/ml solution has an A_{260} nm of 1.4, 24 and 33 respectively.

2.2.5 STATISTICAL ANALYSIS

Effects of gestational age was examined by regression analysis (linear or quadratic) or one way analysis of variance (ANOVA) (Proc GLM, SAS; SAS Institute Inc. Cary, NC, USA) with specific contrasts (Bonferroni). Segmented regression analysis and lines of best

fit were also examined (Cricket Software, Great Valley Corporate Centre, Malvern, PA, USA). Effects of treatment (growth restriction) at each gestational age was assessed by ANOVA with specific contrasts by least squares means (ls means). Relationships between variables were examined by correlation analysis (Proc Corr, SAS) for which data *in utero* was considered both separately and combined with 1 day post-natally.

CHAPTER 3. DETERMINATION OF OVINE IGFBP-4 cDNA SEQUENCE

3.1 INTRODUCTION

Carrier proteins for the IGFs were suggested to exist due to the presence of high molecular weight circulating forms of IGF (Zapf *et al*, 1975) and IGF-binding species have since been isolated from many sources (1.2.1). A symposium, held in 1989, introduced the terminology for the three different types of IGFBP that had been isolated at this time as IGFBP-1, IGFBP-2 and IGFBP-3 (Ballard *et al.*, 1990). In 1989/90, work by P. A. Grant and P. E. Walton, CRC for Tissue Growth and Repair, Adelaide, South Australia, identified an IGFBP in ovine plasma, present at two different molecular weight forms, with a novel N-terminal sequence (Walton *et al.*, 1990). Subsequently, publications by Mohan *et. al.* (1989), and Shimonaka *et al.* (1989), reported the isolation of homologous proteins from bone cell culture medium and adult rat plasma, respectively. Shimasaki *et al.* (1990a), further characterised the cDNA sequence of human and rat forms of this IGFBP, shortly followed by Kiefer *et al.* (1991a), who reported the human cDNA sequence. A second symposium in 1991 agreed on the naming of this IGFBP as IGFBP-4 and two other novel IGFBPs that had since been isolated as IGFBP-5 and IGFBP-6 (Ballard *et al.*, 1992). Comparisons of all the known IGFBP sequences within the one species indicates a striking conservation of sequence (Chapter 1, Figure 1.4 and Figure 1.5). In particular, is the conservation of the position of 18 cysteine residues with the exception of human and rat IGFBP-6 which have 16 and 14 conserved cysteine residues, respectively, and IGFBP-4, which has an additional two cysteine residues. Comparisons of IGFBP sequences between different species again shows strong conservation of sequence, as is indicated in Figure 3.5 for IGFBP-4. Thus, the IGFBPs represent a highly homologous family of proteins that show strong evolutionary conservation.

Prior to commencement of this project and the publication of Shimasaki *et al.* (1990a), it had been a goal to isolate the cDNA sequence for the novel IGFBP purified from sheep plasma by Walton *et al.* (1990). The reverse transcription polymerase chain reaction

(RT-PCR) was to be used to generate a small N-terminal probe with which to screen a sheep cDNA library. Walton and Grant had obtained N-terminal sequence data for ovine IGFBP-4 (oIGFBP-4) and were working to obtain further internal protein sequence. Degenerate 5' and 3' DNA oligomers for PCR were to be designed from this protein sequence data for oIGFBP-4. However, the publication of the human and rat IGFBP-4 DNA sequences (Shimasaki *et al.*, 1990a) allowed consideration of these known IGFBP-4 DNA sequences, which were expected to be highly homologous to oIGFBP-4, for the isolation of an oIGFBP-4 cDNA.

In this study, DNA sequence encoding the mature protein and the 3' non-coding sequence for oIGFBP-4, has been obtained by cDNA library screening and RT-PCR. The DNA and protein sequence is highly homologous to reported sequences for IGFBP-4 from other species. The isolation of oIGFBP-4 cDNA sequence has not only yielded useful comparative sequence data but more importantly provides a suitable probe for analysis of oIGFBP-4 mRNA. The distribution and size of oIGFBP-4 mRNA has been defined, using an isolated IGFBP-4 clone as a probe, in the adult ewe and early (0.3 gestation) and late gestation (near term) ovine fetuses, providing novel data on IGFBP-4 mRNA in the adult and fetus in a large mammalian species.

While this work was in progress Moser *et al.* (1992) published the sequence of bovine IGFBP-4, Gao *et al.* (1993) have presented the structure of the rat IGFBP-4 gene and Mohan *et al.* (1994) and Zazzi *et al.* (1994), have both recently reported abstracts on the structure of the human IGFBP-4 gene.

3.2 MATERIALS AND METHODS

3.2.1 LIBRARIES AND ENZYMES

An ovine liver λgt11 cDNA library was kindly provided by Professor G. Barritt, Department of Medical Biochemistry, Flinders Medical Centre, Bedford Park, South Australia and a SWAJ-2 5' stretch ovine liver cDNA library was a kind gift from Dr. J. O'Mahoney, Centre for Animal Biotechnology, University of Melbourne, Parkville, Vic., Australia. A human fetal liver (first trimester) λgt11 cDNA library was provided by Dr. B.

K. May, Department of Biochemistry, University of Adelaide, Adelaide, South Australia. All libraries were from Clontech Laboratories Inc, Palo Alto, CA, USA. Colony/plaque screen was purchased from Dupont, Boston, MA, USA. Murine Myeloid Leukaemia virus (MMV) Reverse Transcriptase was purchased from Bethesda Research Laboratories, Life Technologies Inc. (BRL), Gaithersburg, MD, USA. Exonuclease III deletion kit, Avian Myeloid Leukaemia Virus (AMV) Reverse Transcriptase and all restriction endonucleases were obtained from Pharmacia, Uppsala, Sweden. Bluescript KS^- plasmid was from Stratagene, La Jolla, CA, USA. *Taq* Thermostable Polymerase was obtained from Bresatec Ltd, Thebarton, South Australia and Pyrostase Thermostable DNA Polymerase was obtained from Molecular Genetic Resources Inc., Tampa, Florida, USA.

3.2.2 DNA OLIGOMERS

All DNA oligomers were purchased from Bresatec Ltd, Thebarton, South Australia. Positions of these primers relative to the DNA sequence for oIGFBP-4 are shown in Figure 3.4.

P15 : (15mer), degenerate oligomer, complementary DNA sequence encoding the protein sequence CCGCG (common to all IGFBPs except IGFBP-6)

5' GCA (A/G)CA (A/G)CC GCA GCC 3'

P29 : (29mer), homologous DNA sequence encoding *Eco*RI site-DEAIHCPP (N-terminal primer)

5' CCG GAA TTC GAC GAA GCC ATC CAC TGC CC 3'

P2435 : (29mer), complementary DNA sequence encoding SRTHEDL-*Eco*RI site

5' CCG GAA TTC GGT CTT CGT GGG TGC GGC TC 3'

P3004 : (28mer), complementary DNA sequence encoding PSDKDE-*Eco*RI site

5' CCG GAA TTC CTC GTC CTT GTC AGA GGG C 3'

P5109 : (30mer), degenerate oligomer, complementary to primer P15 with homologous DNA sequence encoding *Eco*RI site-EPGCGCC (common to all IGFBPs except IGFBP-6)

5' CAG GAA TTC GAG CCG GGC TGC GGC TG(C/T) TG(C/T) 3'

P5394 : (24mer), complementary DNA sequence encoding LGKGM-*Eco*RI site

5' GTA GAA TTC CAT CCC CTT GCC CAG 3'

3.2.3 *E. coli* STRAINS

LE392 *supE44supF58hsdR514galk2galT22metB1trpR55lacY1*

(for screening with SWAJ-2)

Y1090*hsd*R *supFhsdRaraD139ΔlonΔlac*U169*rpsLtrpC*22::Tn*10*(*tet*^{r})pMC9

(for screening with λgt11)

(Sambrook *et al.*, 1989)

3.2.4 PCR AND CLONING OF RAT IGFBP-4 N-TERMINAL SEQUENCE

Approximately 2 µg of total RNA from fetal or maternal sheep liver or rat liver was denatured at 80° C for three minutes with 0.8 µM P15 in 50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.01 % (w/v) gelatin and 500 µM each dNTP. The reaction was cooled to 42° C, 100 U of MMV reverse transcriptase added and incubated at 42° C for one hour. The cDNA was precipitated with 1/10th the volume of 3 M NaOAc, pH 5.2 and two volumes of ethanol, overnight at -20° C. Precipitated cDNA was recovered by centrifugation and resuspended in 200 µl of 0.1 mM EDTA. cDNA (5 µl) was subjected to PCR in 70 mM Tris, pH 8.8, 17 mM $(NH_4)SO_4$, 1 mM β-mercaptoethanol, 7 µM EDTA, 0.15 % (v/v) Triton X-100, 0.02 % (w/v) gelatin, 3 mM $MgCl_2$, 300 µM each dNTP with approximately 0.7 µM P15 and P29 and 2.5 U *Taq* polymerase. The positions of primers P15 and P29 are shown in Figure 3.4. Reactions were cycled at 94° C (1 minute), 58° C (1 minute 20 seconds), 72° C (1 minute), for 35 cycles, followed by a final 5 minute extension at 72° C (Perkin-Elmer Cetus DNA Thermal Cycler, Norwalk, Connecticut, USA).

Products were visualised by 2 % (w/v) agarose mini-gel electrophoresis and stained with ethidium bromide. No products were observed under any reaction conditions from sheep cDNA. An expected 114 bp PCR product was obtained from rat cDNA. This product was gel purified and DNA ends blunted with 2 U T4 DNA polymerase and 200 µM each dNTP in 1 x Superduper buffer (2.1.3) at 37° C for 30 minutes. T4 DNA polymerase was inactivated at 70° C for 15 minutes. The DNA was then digested in 2 X Superduper buffer with 5 U of *Eco*RI at 37° C and ends phosphorylated with 1.5 U T4 Polynucleotide kinase and 1 mM rATP in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$ at 37° C for one hour. DNA was then ligated into approximately three fold excess of *Eco*RI / *Sma*I digested Bluescript KS$^-$ vector, transformed into competent cells (DH5α) and plasmid DNA from resulting colonies prepared and sequenced, as described in section 2.2.1.

3.2.5 cDNA LIBRARY SCREENING

3.2.5.1 Screening ovine and human cDNA libraries with a rat N-terminal probe

The rat N-terminal PCR product was radiolabelled by hot PCR with 25 µCi [α-^{32}P]dATP, 3 µM dATP, 300 µM dTTP, dCTP and dGTP substituted for the dNTP mix described above, using the cloned rat IGFBP-4 PCR product as template DNA. Radiolabelled DNA was purified on a 10 % polyacrylamide (w/v, 25 : 1, acrylamide : bis-acrylamide), 1 X TBE gel and eluted into 0.1 mM EDTA at 37° C overnight. The DNA probe was heat denatured and used to screen both λgt11 ovine liver and human fetal liver cDNA libraries, as below. Approximately 0.5 x 10^6 plaques were screened at a density of around 50,000 plaques per plate. LB, 0.1 % (w/v) maltose, 10 mM $MgSO_4$ (LB + MM) plates (10 plates, 15 cm diameter) were warmed and dried at 37° C. For each plate 300 µl Y1090 overnight culture was inoculated with approximately 50,000 plaque forming units (pfu) of cDNA library and incubated at 37° C for 15 minutes. Infected cells were mixed with 9 ml of melted LB + MM + 0.7 % (w/v) agarose at 42° C and immediately plated. The agarose was allowed to set then plates inverted and grown at 37° C for approximately 6 hours then left overnight at 4° C. Plaques were transferred, in duplicate, to colony/plaque screen membranes (one minute overlay) and filters treated in 1.5 M NaCl, 0.5 M NaOH for

one minute, 1.5 M NaCl, 0.5 M Tris, pH 8.0, two minutes then 2 X SSPE, two minutes. DNA was cross-linked to the membranes with ultra violet (UV) light (UV Strata linker 1800, Stratagene, La Jolla, CA, USA). Filters were prehybridised in 20 % (v/v) deionised formamide, 6 X Denharts solution, 60 mM Tris, pH 7.5, 0.1 % (w/v) SDS, 0.1 % (w/v) sodium pyrophosphate, 0.1 M NaCl, 10 % (w/v) dextran sulphate, 0.05 mg heat denatured salmon sperm DNA at 37° C overnight. The heat denatured probe in 20 % (v/v) deionised formamide (approximately 0.5 x 10^6 cpm/ml) was added to the prehybridisation mix and filters hybridised overnight at 37° C. Filters were washed up to 42° C in 2 x SSC, 0.1 % (w/v) SDS and exposed to x-ray film at -80° C for five days. Four duplicate positives from the ovine cDNA library were selected and subjected to second and third round screening, as above, at plaque densities of 5,000 and 50 respectively. Two duplicate positives from the human fetal cDNA library were selected and subjected to second and third round screening. Lambda DNA was prepared from third round positive plaques, as described below. *Eco*RI inserts were isolated, subcloned into Bluescript KS^- and sequenced, as described in section 2.2.1. The two human clones proved to represent human IGFBP-4 sequences. The four ovine clones were unrelated sequences and were not pursued further.

3.2.5.2 Screening ovine cDNA libraries with a human IGFBP-4 probe

The *Eco*RI insert from the human IGFBP-4 clone, isolated above, was radiolabelled by random priming (2.2.1.6) and used to rescreen the λgt11 ovine liver cDNA library and a SWAJ-2, 5' stretch ovine liver cDNA library. The method was as described above, with the exception of hybridisations at 42° C in 50 % (v/v) deionised formamide. Additionally, the SWAJ-2 vector utilised the cell line LE392. Filters were washed up to 55° C in 2 x SSC, 0.1 % (w/v) SDS and autoradiographed for three days at -80° C. From 16 third round duplicate positives (λgt11) and six positives (SWAJ-2), clones 7, 9 and 10 (λgt11) and 2X (SWAJ-2), were fully characterised. Lambda DNA was prepared, as described below, and digested with appropriate restriction enzymes. *Eco*RI inserts (λgt11) or *Eco*RI/*Xba*I inserts (SWAJ-2), (1.4 kb, 1.6 kb, 1.8 kb and 1 kb, for clones 7, 9, 10 and 2X, respectively) were subcloned into Bluescript KS^- and sequenced. Sequencing compressions were resolved

with deoxyinosine triphosphate. Further subcloning and Exonuclease III deletion series, as described below, were used to fully characterise the clones in both directions. None of the characterised clones contained full length cDNA sequences. The remaining positive clones were shown by insert size, Southern analysis and restriction mapping to also lack 5' sequences.

3.2.6 PREPARATION OF λ DNA

Lambda DNA was prepared according to a method adapted from Sambrook *et al.* (1989). Third round positive plaques were plated on LB + MM plates at high density and plates grown to lysis, overnight at 37° C. The bacteriophage lawn was covered with 3 ml Phage storage buffer (PSB: [10 mM $MgSO_4$, 10 mM Tris, pH 7.4, 100 mM NaCl, 0.01 % (w/v) gelatin]) and bacteriophage eluted at 4° C overnight. Eluate was recovered, a drop of chloroform added to lyse the cells and the high titre bacteriophage stock stored at 4° C. LB + MM (2 ml) was inoculated with 200 µl of Y1090 or LE392 overnight culture and 20 µl of high titre bacteriophage eluate added and grown at 37° C until the culture had lysed (approximately two hrs). At the same time 50 ml LB + MM was inoculated with 500 µl Y1090 or LE392 overnight culture and grown at 37° C. The lysed 2 ml culture was then added to the 50 ml cell culture and grown at 37° C until the cells were lysed (approximately two hrs). A drop of chloroform was added and cellular nucleic acid digested with 0.7 mg/ml RNaseA and 0.7 mg/ml DNase I at 4° C overnight. The NaCl concentration was raised to 1 M by the addition of 6.5 % (w/v) NaCl to dissociate bacteriophage particles from cell membrane fragments and the cell debris pelleted at 12,000 x g for 15 minutes at 4° C. The supernatant was recovered and centrifugation repeated, as above. Clarified supernatant was transferred to a fresh tube and bacteriophage particles pelleted by centrifugation at 18,000 x g for three hours at 4° C. Pellets were drained and resuspended in 500 µl PSB. Protein was digested in 0.02 M EDTA, pH 8.0, 0.2 % (w/v) SDS with 0.05 mg /ml Proteinase K at 65° C for one hour. The solution was extracted twice with an equal volume of 50 % (v/v) Tris-saturated phenol, 50 % (v/v) chloroform and the aqueous phase precipitated with 1/10th the volume of 3 M NaOAc, pH 5.2 and two volumes of ethanol at -

20° C for one hour. DNA was recovered by centrifugation, washed twice with 70 % (v/v) ethanol and resuspended in 100 µl of water. Recovered λ DNA was analysed by agarose mini-gel electrophoresis.

3.2.7 EXONUCLEASE III NESTED DELETION SERIES

Exonuclease III nested deletion series were generated from clone 9 and 10 (λgt11) to fully characterise the DNA sequence bidirectionally. The cloned DNA was uniquely restricted in the polylinker region on one side of the DNA insert with two different restriction enzymes. The enzyme cutting closest to the insert must leave a blunt end or 5' overhang, while the distal cutting enzyme must leave a 3' overhang. The enzymes chosen were *Kpn*I (distal) and *Eco*RV (proximal). Approximately 2 µg of DNA was cut with both enzymes and the linearised DNA purified on a Sepharose CL-6B spin column (2.2.1.4). Deletions of approximately 200 bp were generated using a Pharmacia Exonuclease III nested Deletion Kit, according to the manufacturers instructions, using the supplied buffers and enzymes. Briefly, 20 µl of DNA was equilibrated with an equal volume of 2 x Exo III buffer (Tris, pH 8.0, $MgCl_2$) at 25° C for three minutes. A 2 µl sample was removed (t=0) and added to S1 nuclease mix (KOAc, pH 4.6, NaCl, $ZnSO_4$, glycerol, S1 nuclease enzyme) on ice. Exonuclease III enzyme (1 µl) was added to the DNA mix and incubated at 25° C, taking 2 µl samples at six minute intervals and adding directly to the S1 nuclease mix, on ice. After 20 samples were taken all tubes were incubated at room temperature for 30 minutes. One microlitre of S1 nuclease stop mix (Tris, EDTA) was added and S1 nuclease inactivated at 65° C for 10 minutes. An aliquot from each time point (2 µl) was checked on a 1 % (w/v) agarose mini-gel to analyse the extent of deletion. The remaining blunt ended samples were ligated at 14° C overnight, transformed into competent cells (DH5α), and plasmid DNA isolated (2.2.1). The extent of the deletion was analysed by restriction enzyme digestion and the DNA from appropriate clones sequenced as described in 2.2.1.5.

3.2.8 PCR AND CLONING OF OVINE IGFBP-4 N-TERMINAL SEQUENCE

N-terminal sequences of ovine IGFBP-4, not represented by cDNA library clones, were obtained by Reverse Transcription-PCR (RT-PCR). Total RNA (20 µg) from a 60 day fetal ovine kidney was reverse transcribed with AMV-Reverse Transcriptase at 42° C for 60 minutes in 50 mM Tris, pH 8.3, 30 mM KCl, 80 mM $MgCl_2$ and 400 µM each dNTP with P2435 (PCR1) or P3004 (PCR2) in a total volume of 50 µl. The positions of primers P2435 and P3004 are indicated in Figure 3.4. cDNA was ethanol precipitated and resuspended in 200 µl water. PCR was performed in 50 mM Tris, pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.005 % (w/v) BSA, 250 µM each dNTP, 0.5 µM each primer, 2 U Pyrostase Thermostable DNA Polymerase, with 0.5 µl of appropriate reverse transcription reaction in a final volume of 20 µl. Reactions were overlaid with paraffin oil and cycled in a two step procedure : 94° C (40 seconds), 58° C (1 minute), 72° C (1 minute), for 25 cycles, 94° C (40 seconds), 60° C (1 minute), 72° C (1 minute), for five cycles, 72° C (15 minutes) (Perkin-Elmer Cetus DNA Thermal Cycler, Norwalk, Connecticut, USA).

PCR 1 A degenerate primer was synthesised with DNA sequence homologous to that encoding the conserved protein sequence EPGCGCC of all IGFBPs (P5109). Using the cDNA from reverse transcription with P2435 and oligomers P5109 and P3004 a PCR fragment of 230 bp was amplified.

PCR 2 Using the sequence data obtained from PCR1, the complementary oligomer P5394 was designed. From N-terminal protein sequence data (Walton *et al.*, 1990) a primer of homologous DNA sequence, P29, was synthesised. Using these two primers and cDNA from reverse transcription with P3004, a PCR fragment of 150 bp was generated.

The positions of primers for PCR1 and 2 are indicated in Figure 3.4. Products from PCR1 and 2 were cloned into Bluescript KS^- using *Eco*RI restriction endonuclease sites designed into the 5' ends of the PCR primers. DNA sequence was determined from at least three independent clones from each PCR reaction to account for PCR-induced errors.

3.2.9 SEQUENCE ANALYSIS

DNA, RNA and protein sequences were analysed and compared using the Genetics Computer Group (GCG, University of Wisconsin) Sequence Analysis Software Package (Devereux *et al.*, 1984). The programs BESTFIT (Smith and Waterman, 1981), GAP (Needleman and Wunsch, 1970) TRANSLATE and PEPTIDE STRUCTURE (Jameson and Wolf, 1988) were used for sequence comparisons and structural predictions. The FOLD (Zucker and Stiegler, 1981) program was used for RNA secondary structure predictions.

3.3 RESULTS

3.3.1 N-TERMINAL SEQUENCE OF THE RAT IGFBP-4 PCR PRODUCT

The reverse transcription polymerase chain reaction (RT-PCR) was used to generate a N-terminal IGFBP-4 probe. This reaction was only successful using rat cDNA and no products were generated from RT-PCR off ovine RNA. The rat PCR product was cloned and sequenced (Figure 3.1). The sequence obtained was identical to that reported by Shimasaki *et al.* (1990a) with the exception of two base pairs that were unresolved due to a sequencing compression and a base substitution in the P15 primer region, introduced by the degeneracy of this oligomer.

3.3.2 PARTIAL DNA SEQUENCE OF A HUMAN IGFBP-4 cDNA CLONE

The rat IGFBP-4 clone shown in Figure 3.1 was used as a DNA template for radiolabelled PCR to generate a probe to screen both ovine and human cDNA libraries. Only positive clones for human IGFBP-4 were obtained. These were partially characterised by DNA sequencing to confirm the identity as human IGFBP-4. The partial DNA sequence obtained from one human IGFBP-4 cDNA clone is presented in Figure 3.2 and is identical to the reported sequence for human IGFBP-4 with one residue unresolved due to sequencing compressions (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a).

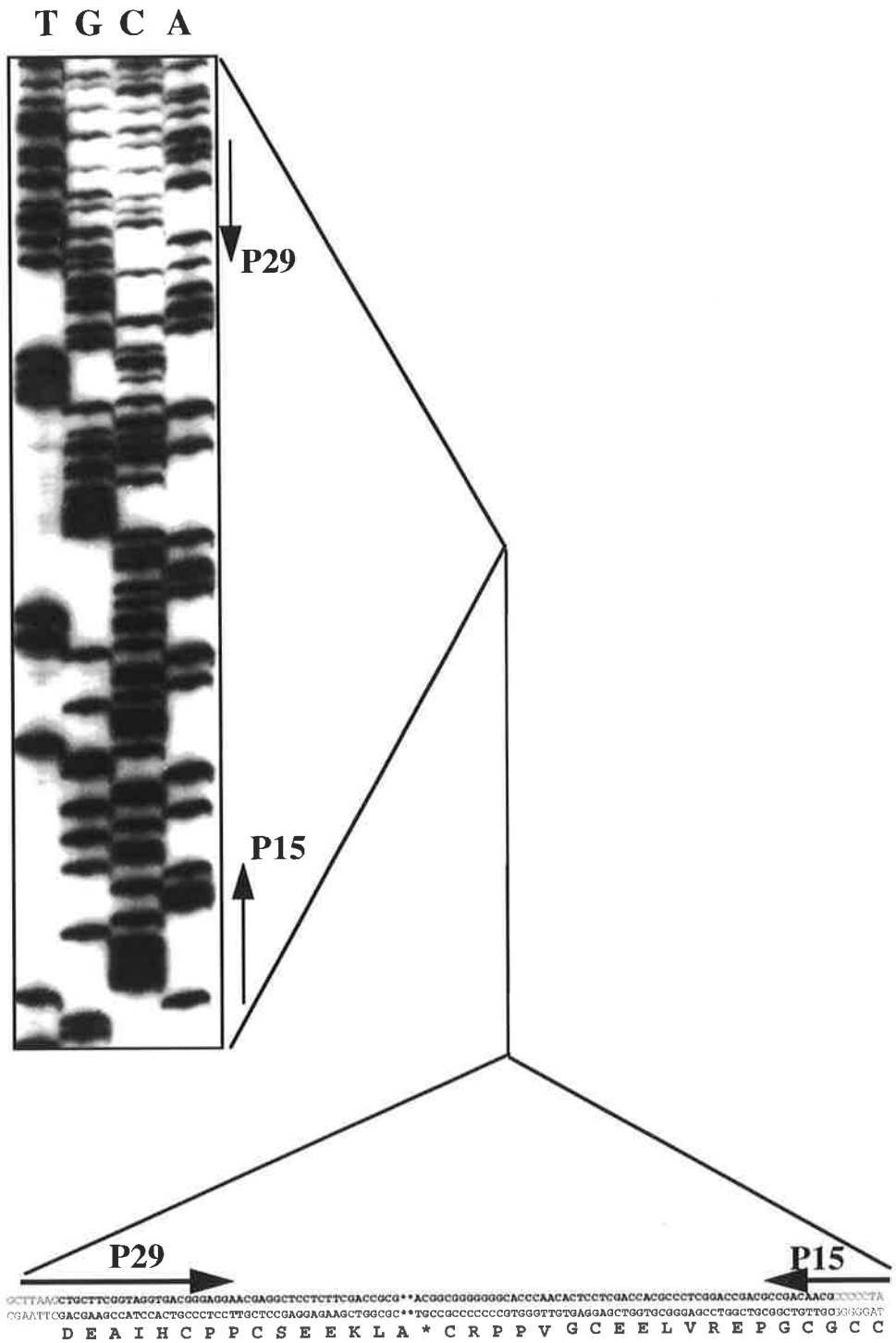


FIGURE 3.1 SEQUENCE ANALYSIS OF RAT IGFBP-4 PCR PRODUCT. A PCR product encompassing the N-terminal sequence of the mature rat IGFBP-4 protein was generated from rat liver RNA, using primers P29 and P15, as described in 3.2.4. The PCR product was cloned and sequenced (3.2.4) and showed identity to the published sequence for rat IGFBP-4 (Shimasaki *et al.*, 1990a). * = base or amino acid not determined due to sequencing ambiguities.

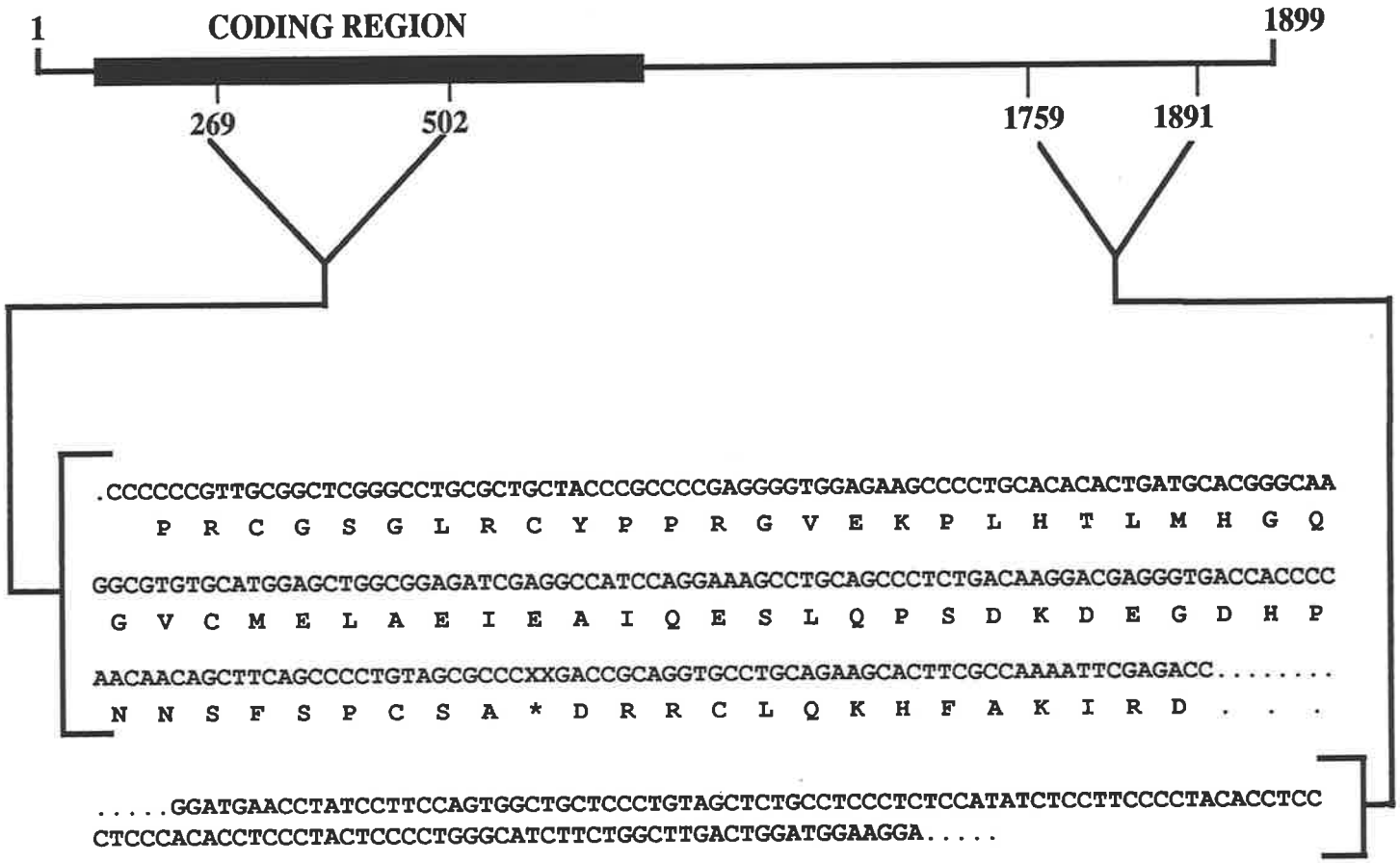


FIGURE 3.2 PARTIAL DNA SEQUENCE OF HUMAN IGFBP-4 CLONE. A human IGFBP-4 clone was isolated by cDNA library screening, with the rat IGFBP-4 PCR product, and characterised by partial sequence analysis. DNA sequencing of the 3' end and internal coding region positively identified the clone as human IGFBP-4. Sequence is identical to published data (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a). X = base not determined, * = amino acid not determined. Numbering refers to the published DNA sequence of human IGFBP-4 by Shimasaki *et al.* (1990a).

3.3.3 cDNA AND PROTEIN SEQUENCE OF OVINE IGFBP-4

Using a combination of cDNA library screening, with the human IGFBP-4 cDNA clone as a probe, and RT-PCR, partial clones representing oIGFBP-4 were generated (Figure 3.3). From these clones the DNA sequence encompassing the full mature protein coding region and 3' non-coding end for oIGFBP-4 was deduced (Figure 3.4). The coding region of the DNA sequence has 86, 94, and 98 % identity to rat, human and bovine IGFBP-4, respectively (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a; Moser *et al.*, 1992). There is also a high degree of identity (68 and 74 %) over regions of the 3' non-coding end with rat and human IGFBP-4, respectively. Bovine and oIGFBP-4 show a remarkable 96 % identity over the 3' non-coding region, although there are an additional 79 bases between bases 1143 and 1222 and seven bases between 1276 and 1283 in the oIGFBP-4 DNA sequence. In contrast to bovine IGFBP-4, clone 9 was polyadenylated although clone 7 has the same 3' end but lacked a poly A tail. There is no obvious upstream consensus polyadenylation sequence, although the region is highly A/T rich.

The DNA sequence obtained for oIGFBP-4 was translated and compared to rat, human and bovine IGFBP-4 protein sequence (Figure 3.5). As expected, the protein sequence is highly conserved with 90, 96 and 98 % identity between rat, human and bovine, respectively. Figure 3.5 illustrates the three amino acid positions where bovine and oIGFBP-4 differ, whereas nine and 22 positions are indicated for human and rat IGFBP-4, respectively. Many of the amino acid changes observed were conservative raising the protein homology to 95, 97 and 98 %, for rat, human and bovine IGFBP-4, respectively. Computer structural predictions (GCG, PEPTIDESTRUCTURE, Figure 3.6) suggest oIGFBP-4 to be a hydrophilic protein with a potential N-linked glycosylation site at residue 104. Results also predict β-sheets, as determined by the method of Chou and Fasman (1978) or Garnier *et al.* (1978), in the N-terminal region of IGFBP-4 with several α-helices in the remainder of the protein.

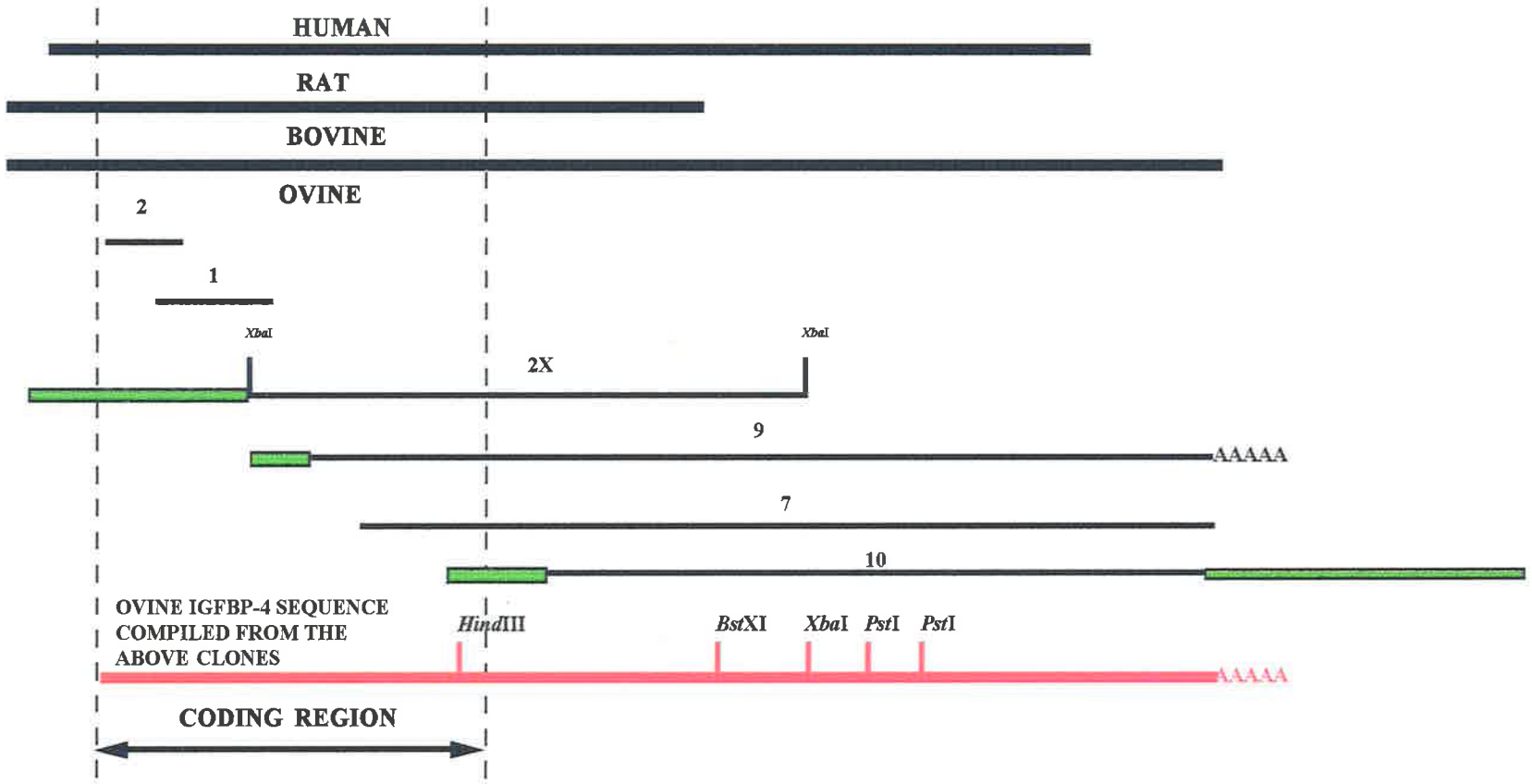


FIGURE 3.3 SCHEMATIC REPRESENTATION OF PARTIAL OVINE IGFBP-4 CLONES. Partial oIGFBP-4 clones were obtained by cDNA library screening (10, 7, 9 or 2X), or generated by PCR off ovine fetal kidney RNA (1 and 2), as described in 3.2.8 The positions of these clones are shown relative to schematic representations of human, rat (Shimasaki *et al.*, 1990a) or bovine IGFBP-4 (Moser *et al.*, 1992). Regions highlighted in green indicate unrelated sequences due to library cloning artifacts. The complete sequence encompassing the 3' non-translated region and mature protein coding region compiled from the partial clones, is schematically indicated in red. The position of restriction enzyme sites used for subcloning are indicated. The *Xba*I site at the 5' end of clone 2X is a library cloning artifact. Clones 7 and 9 have the same 3' end sequence, although clone 9 also contains a poly A tail.

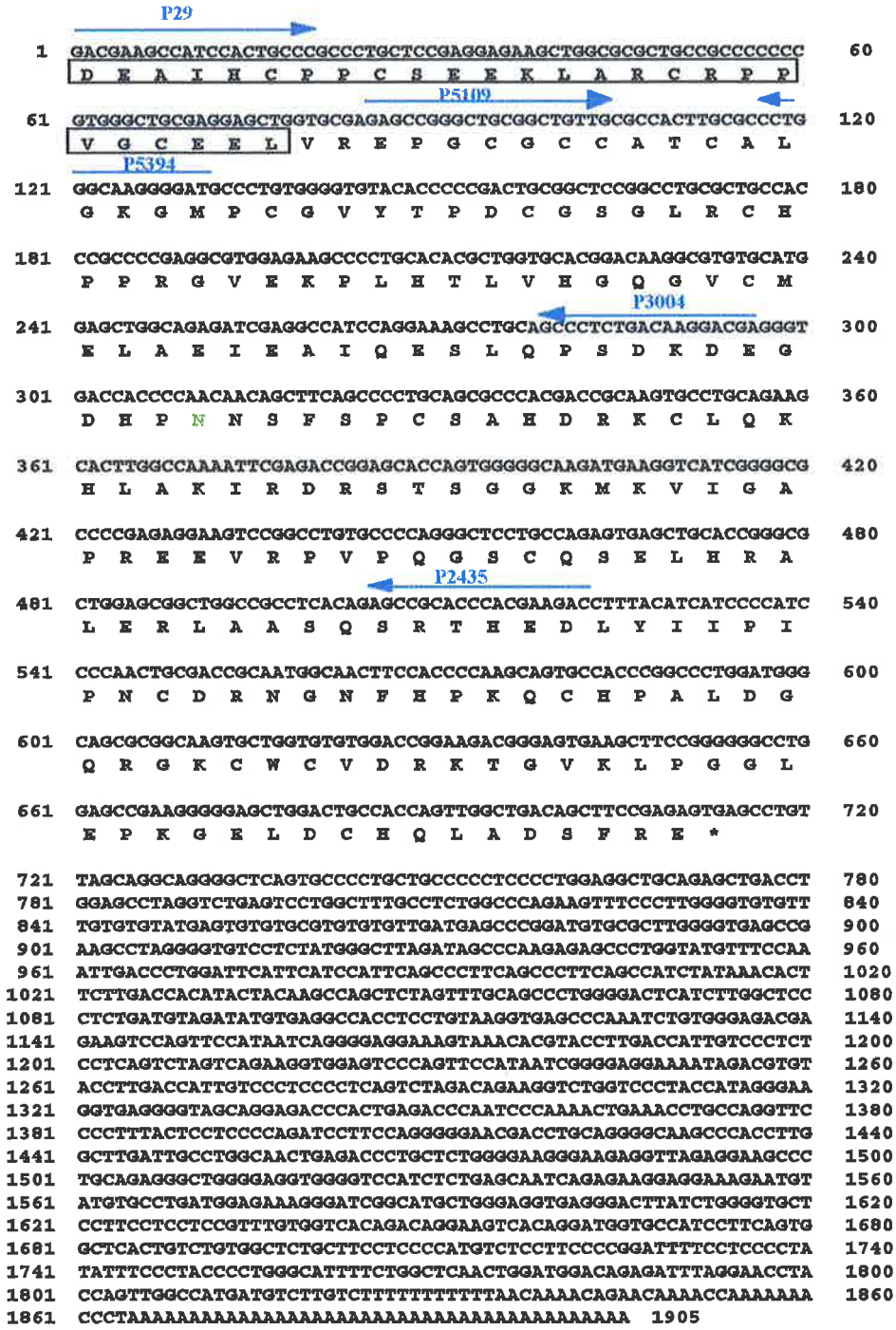


FIGURE 3.4 DNA SEQUENCE OF OVINE IGFBP-4. DNA sequence from the partial oIGFBP-4 clones illustrated in Figure 3.3 was determined (2.2.1.5) and data compiled. Complete DNA sequence encompassing the 3' untranslated end and the mature protein coding region is shown. The translation product for oIGFBP-4 is also shown. The positions of primers used for reverse transcription or PCR are indicated. The N-terminal protein sequence determined by collaborators Walton and Grant, is boxed. The potential N-linked glycosylation site at amino acid position number 104 is indicated in green.

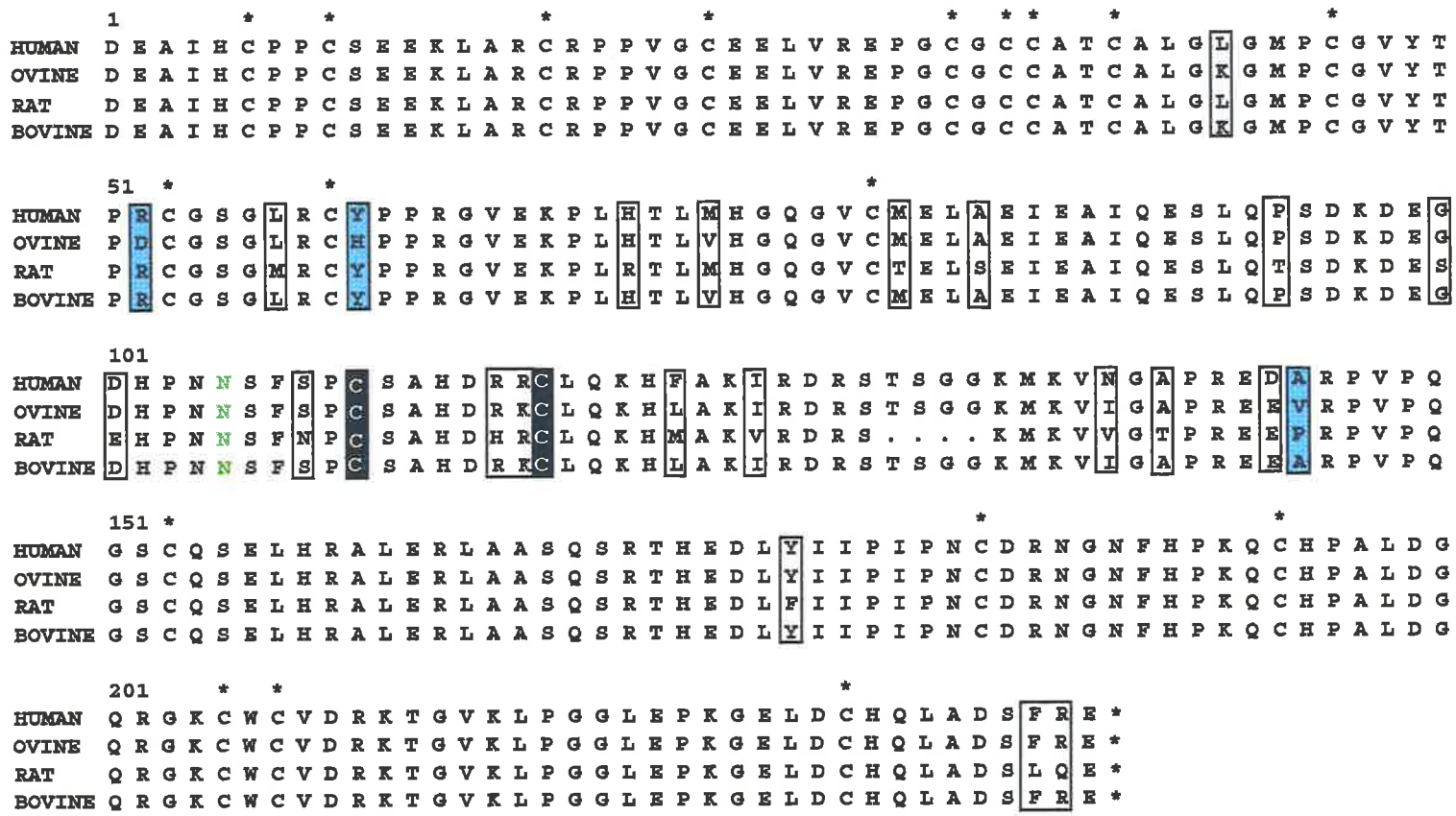


FIGURE 3.5 ALIGNMENT OF THE 4 PROTEIN SEQUENCES FOR IGFBP-4. Mature protein sequence for oIGFBP-4 is compared to sequences for human, rat (Shimasaki *et al.*, 1990a, Kiefer *et al.*, 1991a), and bovine IGFBP-4 (Moser *et al.*, 1992). The 18 cysteine residues common to all IGFBPs, except IGFBP-6, are indicated by *. The two cysteine residues unique to IGFBP-4 are highlighted by black boxes. Positions of amino acid substitutions in any IGFBP-4 sequence are boxed. Positions where ovine and bovine IGFBP-4 differ are highlighted in blue. The potential N-linked glycosylation site at amino acid 104, is indicated in green.

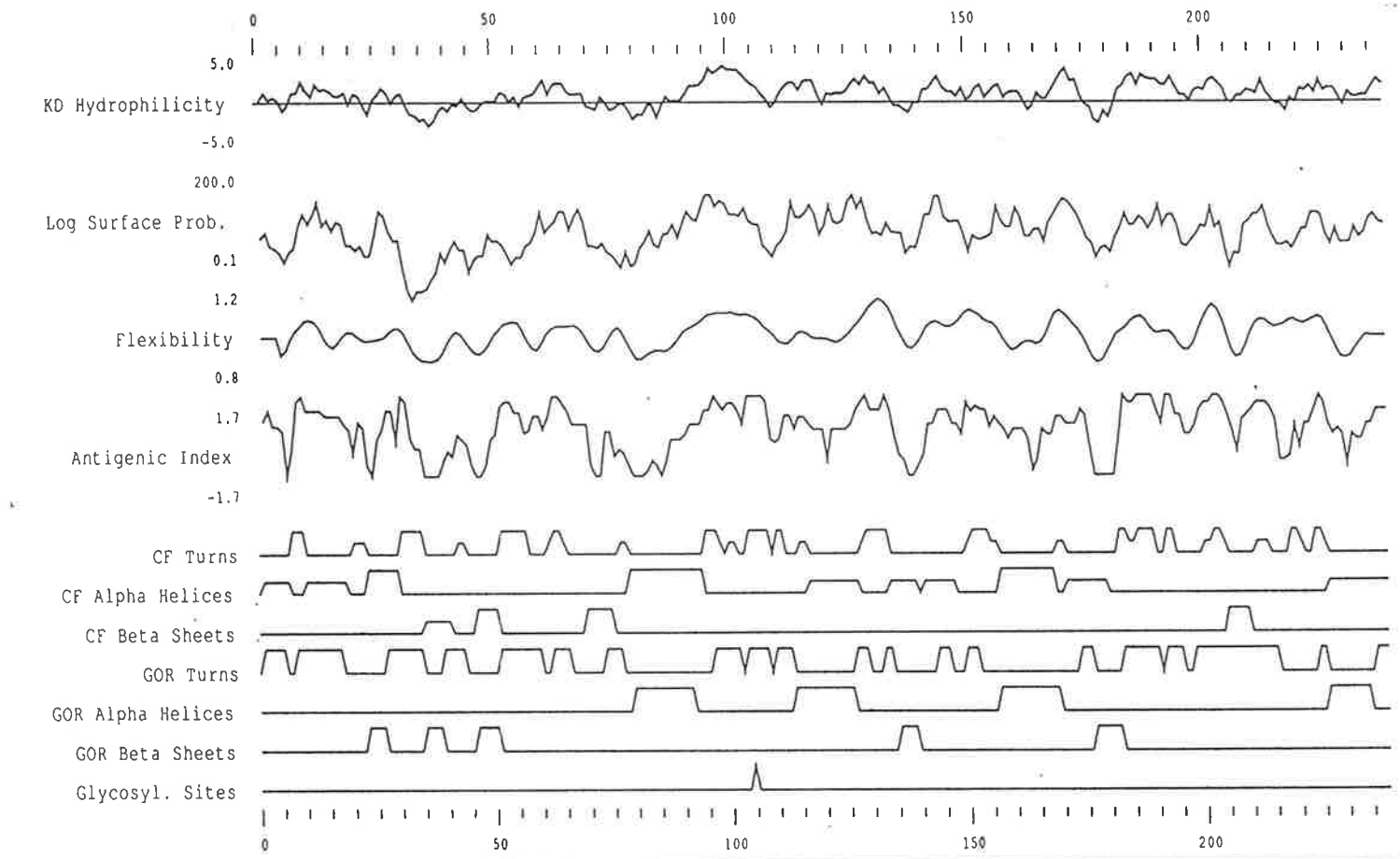


FIGURE 3.6 PREDICTED PROTEIN STRUCTURE FOR OVINE IGFBP-4. Mature protein sequence of oIGFBP-4 was subjected to computer predictions of secondary structure using the GCG programme PEPTIDE STRUCTURE (Jameson and Wolf, 1988). KD = Kyte and Doolittle (1982) hydrophilicity determination. Surface probability was determined by the method of Emini *et al.*, 1985. Antigenic index was determined according to Jameson and Wolf, (1988). Secondary structure was predicted according to Chou and Fasman, (1978) (CF) or Garnier *et al.*, (1978) (GOR).

3.3.4 NORTHERN ANALYSIS OF OVINE IGFBP-4 mRNA

Using clone 2X obtained from the cDNA library screening, the size and distribution of oIGFBP-4 mRNA was assessed by Northern analysis (Figure 3.7A to C). A major transcript of 2.6 kb was detected in both adult and fetal tissues, in early and late gestation. In addition, two minor transcripts of 2.1 and 1.8 kb were also detected. These sizes were determined relative to the migration of an RNA ladder. The size of 18S rRNA by this method was estimated to be approximately 2.0 kb compared to the cited size of human 18s rRNA of 2.3 kb (Sambrook *et al.*, 1989). Steady state IGFBP-4 mRNA levels were high in the kidney ≥ liver > lung and low in the heart from the adult ewe (Figure 3.7A). In the late gestation fetus (145 days gestation, term : approximately 150 days), IGFBP-4 mRNA is low in the heart, placenta, skin, muscle (tibialis or quadraceps) and more abundant in the liver, kidney, heart, lung and adrenal (Figure 3.7B). In contrast, IGFBP-4 mRNA is readily detectable in the 45 day (0.3 gestation) fetal heart and is abundant in the liver, kidney, lung and placenta with lower levels in the brain (Figure 3.7C). Filters were also probed for IGFBP-2, which, in contrast to the more ubiquitous presence of IGFBP-4 mRNA, shows transcripts in many tissues in early gestation but IGFBP-2 mRNA is restricted to the liver and kidney in the late gestation fetus or adult ewe (Figure 3.7A to C). Autoradiographs were scanned, values normalised for rRNA loading (relative to 18s rRNA) and expressed as a percentage of adult ewe liver electrophoresed on each gel. Figure 3.8 shows the comparison of IGFBP-2 and IGFBP-4 mRNAs in the adult ewe and early and late gestation fetus which suggests IGFBP-4 mRNA levels to be regulated in a tissue and developmental specific manner.

3.4 DISCUSSION

Results described in this chapter present sequence data for oIGFBP-4 obtained from partial oIGFBP-4 cDNA clones generated by library screening and RT-PCR. Initial isolation of cDNA clones representing the N-terminal sequences of oIGFBP-4 proved to be difficult using cDNA library screening. The isolation of partial clones missing 5' sequences from cDNA libraries is not uncommon. However, even a commercially available 5' stretch

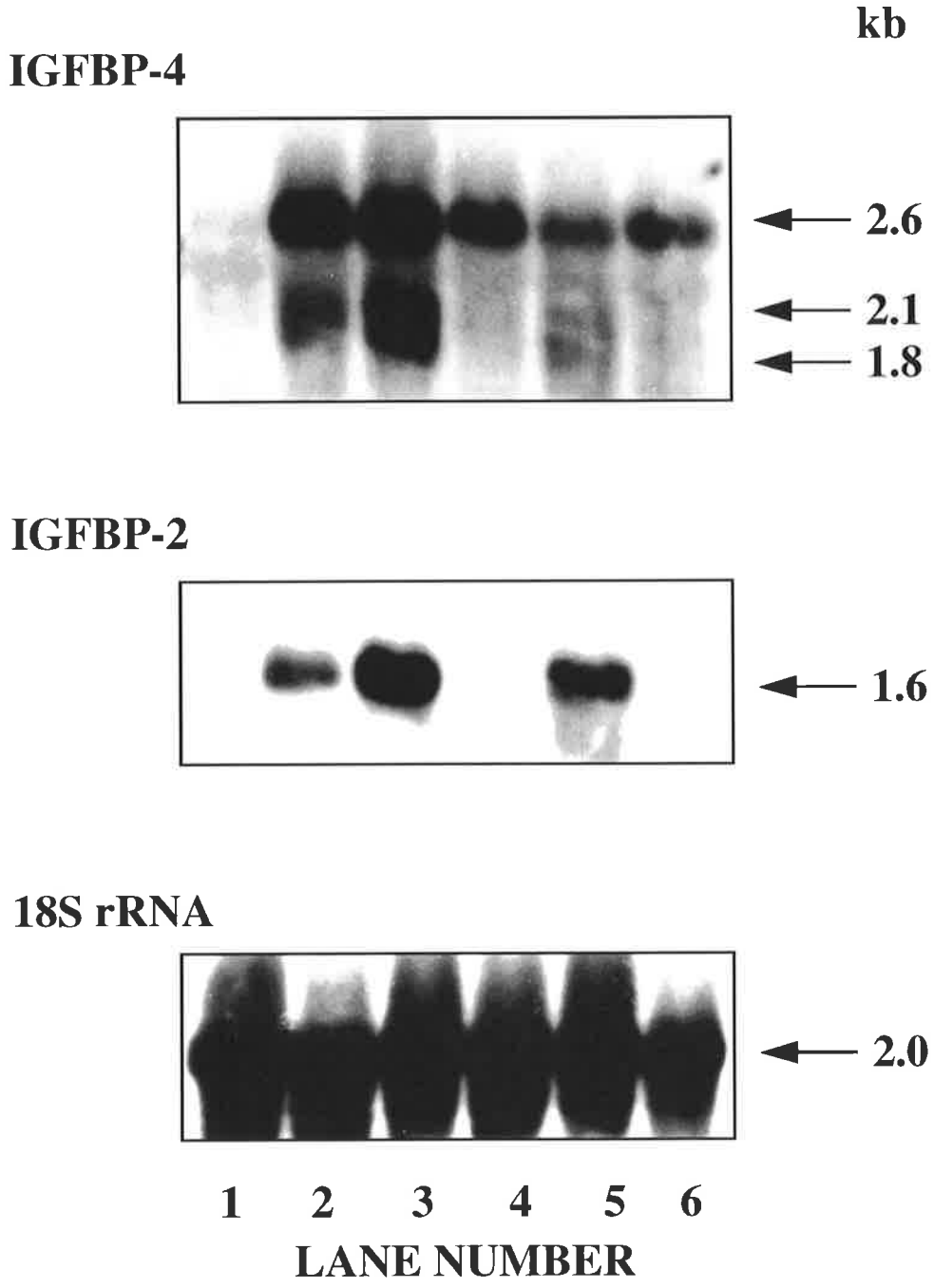
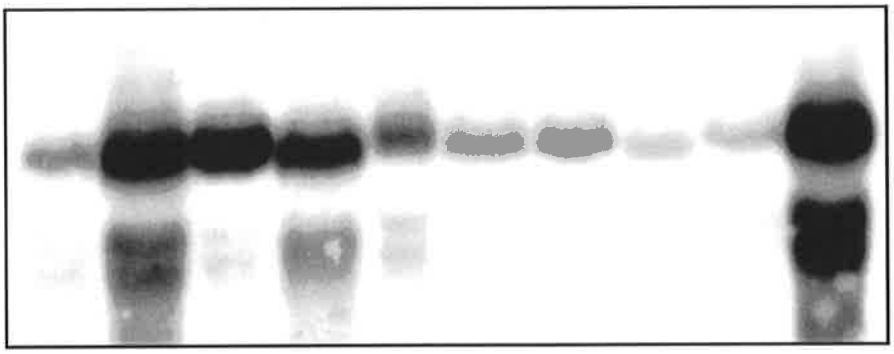
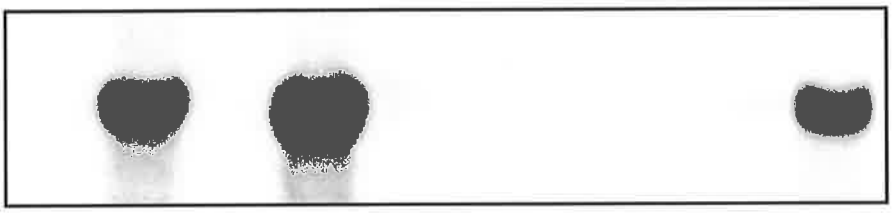


FIGURE 3.7A NORTHERN ANALYSIS OF IGFBP-2 AND IGFBP-4 mRNA IN THE ADULT EWE. RNA was extracted and subjected to Northern analysis (2.2.2) and filters probed for IGFBP-4. Filters were stripped in boiling water and reprobed with IGFBP-2 as a comparison, and 18S rRNA to account for RNA loading. Lane 1 = heart; Lane 2 =kidney; Lane 3 = liver; Lane 4 = lung; Lane 5 = 120 day fetal liver; Lane 6 = adult rat liver. Estimated sizes of mRNA transcripts are indicated.

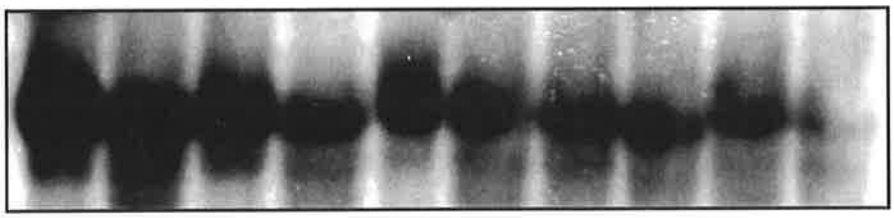
IGFBP-4



IGFBP-2



18S rRNA

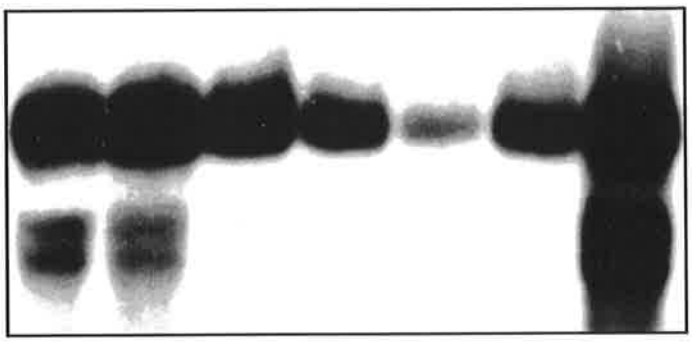


1 2 3 4 5 6 7 8 9 10

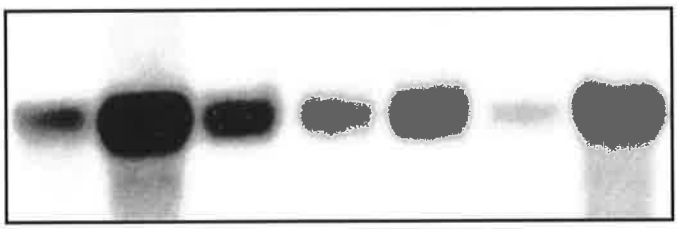
LANE NUMBER

FIGURE 3.7B NORTHERN ANALYSIS OF IGFBP-2 AND IGFBP-4 mRNA IN THE 145 day, LATE GESTATION SHEEP FETUS. RNA was extracted and subjected to Northern analysis (2.2.2) for IGFBP-4. Filters were stripped in boiling water and reprobed with IGFBP-2 as a comparison, and 18S rRNA to account for RNA loading. Lane 1= heart; Lane 2 = liver; Lane 3 = lung; Lane 4 = kidney; Lane 5 = adrenal; Lane 6 = quadraceps muscle; Lane 7 = tibialis muscle; Lane 8 = skin; Lane 9 = cotyledon; Lane 10 = adult ewe liver.

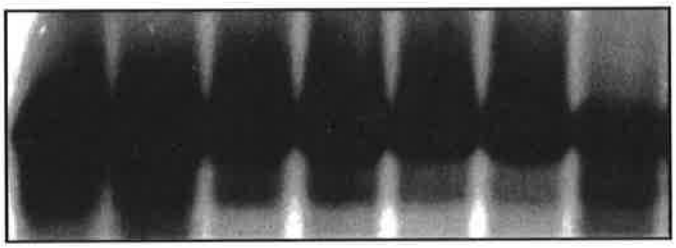
IGFBP-4



IGFBP-2



18S rRNA



1 2 3 4 5 6 7

LANE NUMBER

FIGURE 3.7C NORTHERN ANALYSIS OF IGFBP-2 AND IGFBP-4 mRNA IN THE 45 day, EARLY GESTATION FETAL SHEEP. RNA was extracted and subjected to Northern analysis (2.2.2) for IGFBP-4. Filters were stripped in boiling water and reprobed for IGFBP-2 as a comparison, and 18S rRNA to account for RNA loading. Lane 1 = heart; Lane 2 = liver; Lane 3 = lung; Lane 4 = kidney; Lane 5 = brain; Lane 6 = cotyledon; Lane 7 = adult ewe liver.

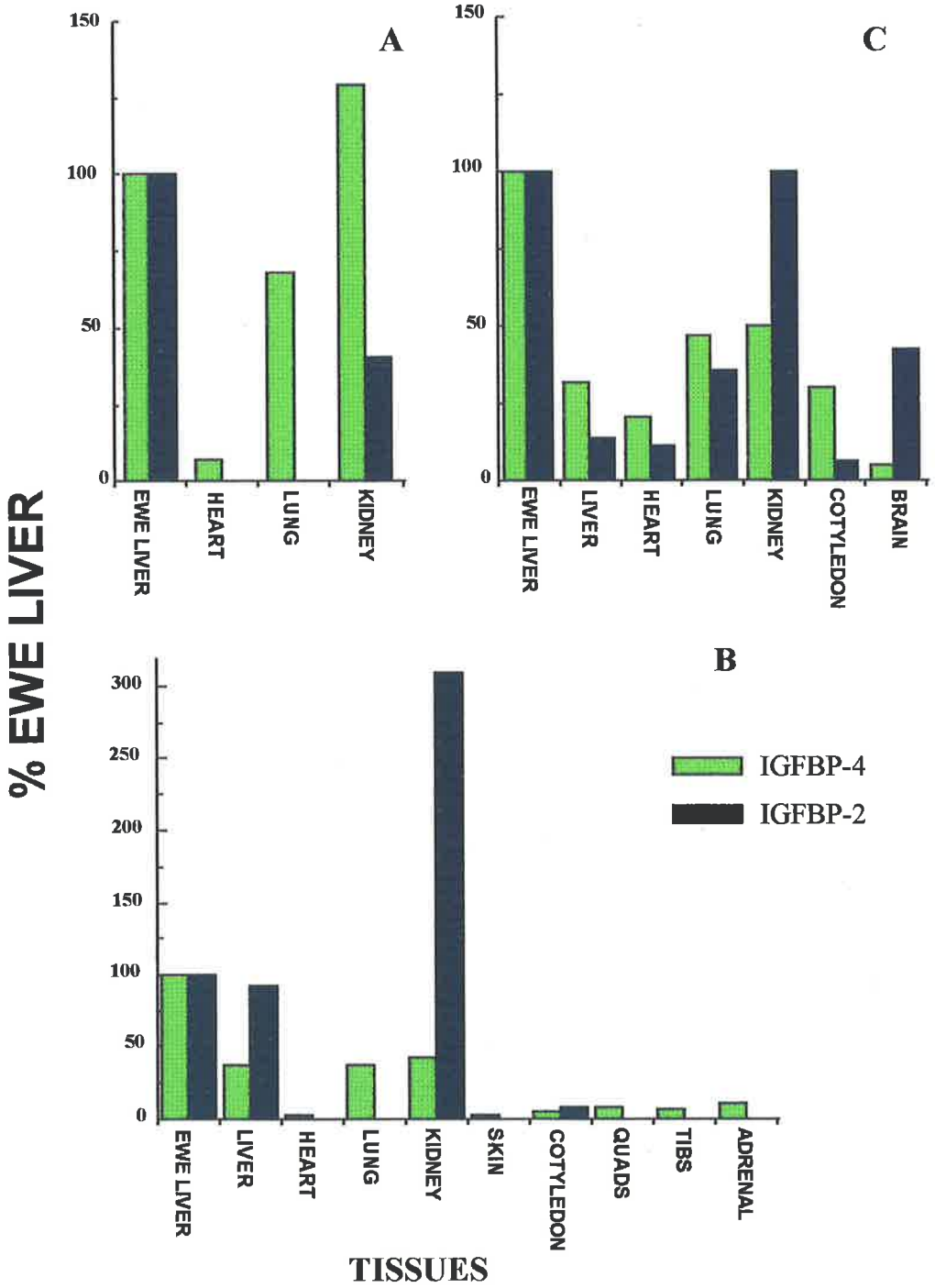


FIGURE 3.8 TISSUE DISTRIBUTION OF IGFBP-2 AND IGFBP-4 mRNA. Northerns shown in Figure 3.7A to C were quantitated by laser densitometry, normalised for RNA loading by expressing as a percentage of 18S rRNA and standardised against the value for the ewe liver sample electrophoresed on each gel. A = adult ewe, B = 145 days gestation ovine fetus, C= 45 days gestation ovine fetus.

library, reportedly enhanced for obtaining 5' sequences, contained no detectable clones with oIGFBP-4 N-terminal sequences. This may be due the quality of the ovine libraries commercially available or strong RNA secondary structure in the 5' region of oIGFBP-4. Computer predictions of RNA secondary structure (GCG, FOLD) for oIGFBP-4 suggest extensive base pairing and stem loop structures over the first 1 kb of sequence. The predicted structure over the most 5' 400 bp of sequence is shown in Figure 3.9 and has a free energy value of -173.2 kcal/mole, suggesting a stable structure. In comparison, the corresponding 400 bp of sequence for rat IGFBP-4 also shows extensive base pairing and has a higher free energy value of -154.6 kcal/mole and is thus a less stable structure (data not shown). The termination points of the clones isolated by library screening and the positions of primers used for PCR are indicated in Figure 3.9. It is possible that this RNA structure caused premature termination of the extending RNA dependent DNA polymerase during cDNA synthesis, thus producing only partial cDNA library clones and shortened templates for PCR, lacking 5' DNA sequences. Additionally, the high G-C content of the 5' end of the IGFBP-4 coding sequence and the frequent occurrence of sequencing compressions over this region are suggestive that the DNA template in this region may also be highly structured.

However, the apparent structural problem at the 5' end of the IGFBP-4 mRNA transcript was overcome by reverse transcribing from priming sites close to the 5' end. Thus, the remaining DNA sequence of oIGFBP-4, encoding the mature IGFBP-4 protein region, was obtained by RT-PCR. Several clones from each PCR reaction were sequenced to identify any PCR-induced errors and ensure the sequence obtained was correct. Attempts at obtaining further 5' sequences encoding the oIGFBP-4 leader peptide and 5' untranslated regions were made using genomic screening and Rapid Amplification of cDNA Ends-PCR (RACE-PCR). RACE-PCR involves generating a 5' priming site of known sequence at the 3' end of the cDNA transcript followed by PCR amplification with a specific internal 3' primer, the basis of which is illustrated in Figure 3.10. RACE-PCR was attempted using two different methods to produce a 5' priming site. cDNA was capped at the 3' end using deoxynucleotidyl terminal transferase, a template independent DNA

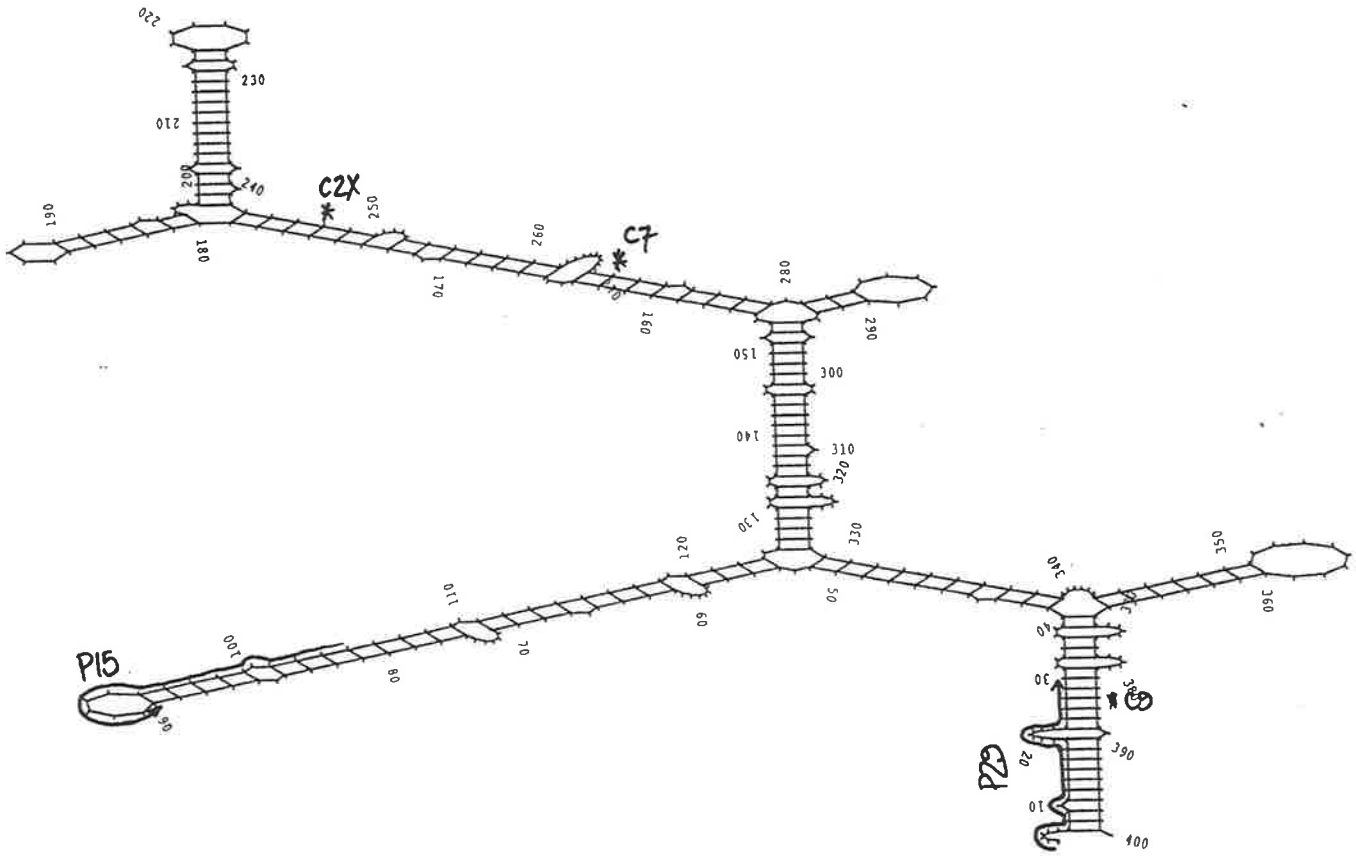


FIGURE 3.9 PREDICTED STRUCTURE OF OVINE IGFBP-4 RNA. The most 5' 400 bp of DNA sequence for oIGFBP-4 was subjected to RNA secondary structure prediction according to the method of Zucker and Stiegler, (1981), using the GCG program, FOLD. The most stable structure is shown and is predicted to have a free energy value of -173.2 kcal/mole. The 5' termination positions of clones isolated by cDNA library screening and the positions of primers used for PCR are indicated. As a comparison, the RNA structure of rat IGFBP-4 over the same sequence is predicted to have a free energy value of -154.6 kcal/mole.

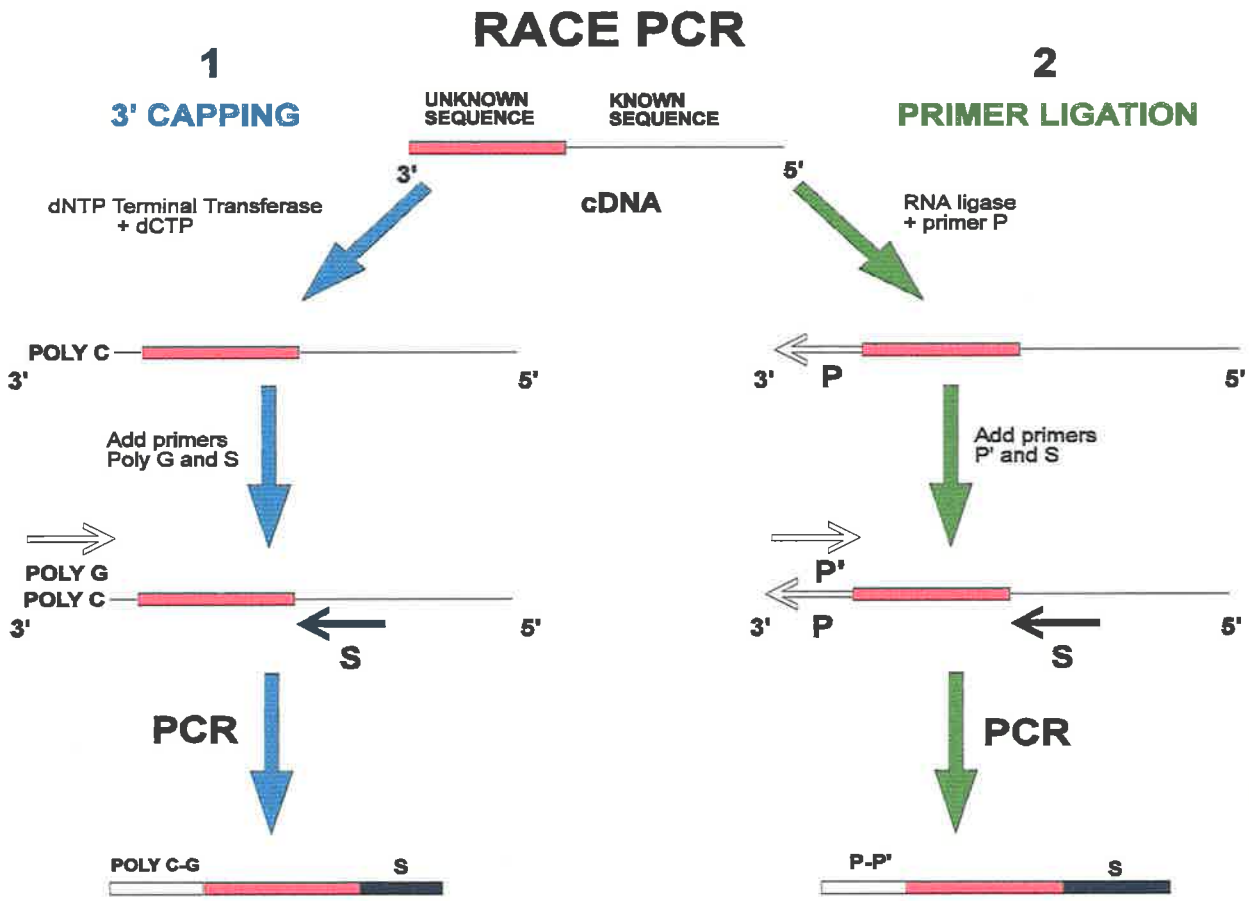


FIGURE 3.10 RAPID AMPLIFICATION OF cDNA ENDS - PCR. RACE-PCR was attempted to try and isolate further unknown 5' sequences for oIGFBP-4, using two alternative methods for generating a novel 5' priming site. **1.** The 3' cDNA end was capped with the enzyme deoxynucleotidyl terminal transferase (a template independent DNA polymerase) and dNTPs. PCR was then performed with a complementary poly dN'TP primer (5') and an oIGFBP-4 specific 3' primer (S). **2.** A unique primer (P) was ligated to the 3' end of the cDNA transcript with RNA ligase. PCR was then performed with a primer complementary to the ligated primer (P') and an oIGFBP-4 specific 3' primer (S). Although the initial steps in both of these methods were successful, neither method resulted in IGFBP-4 specific PCR products.

polymerase, (Boehringer Mannheim, Germany), and dCTP. Poly GTP oligomers were then used as 5' primers with an IGFBP-4 specific 3' oligomer. In the second method, a specific oligomer was ligated to the 3' cDNA end using RNA ligase, followed by PCR with the complementary DNA oligomer (5') and an IGFBP-4 specific 3' oligomer. Neither of the RACE-PCR strategies or genomic screening was successful for isolating the 5' signal sequence and untranslated region of oIGFBP-4 and, in the interest of pursuing results in more promising areas, the search for further 5' sequences was abandoned.

The DNA sequence obtained from the partial clones was compiled and compared to the known sequences for rat, human and bovine IGFBP-4. DNA sequences were very similar, showing 86, 94 and 98 % identity over the coding region to rat, human and bovine IGFBP-4, respectively (Shimasaki *et al.*, 1990a; Kiefer *et al.*, 1991a; Moser *et al.*, 1992). There is also strong homology of the 3' non-coding region between different species, especially between ovine and bovine IGFBP-4. This is suggestive of conservation of 3' regulatory elements that may be involved in processes such as determination of mRNA stability or translatability. Comparison of mature protein sequences across species also shows a high degree of sequence identity, (90, 96 and 98 %, for rat, human and bovine IGFBP-4, respectively), particularly between ovine and bovine IGFBP-4. In most cases where amino acids were substituted the resulting changes were conservative, suggesting strong structural and functional restriction of protein sequence. The particularly strong sequence identity between ovine and bovine IGFBP-4, in comparison to the lower degree of conservation relative to human or rat IGFBP-4, reflects the close evolutionary relationship of sheep and cattle, which are both ungulates.

Computer generated structural predictions suggest oIGFBP-4 to be a hydrophilic protein with a potential N-linked glycosylation site at position 104 of the mature protein. This has previously been suggested for rat and human IGFBP-4 (Shimasaki *et al.*, 1990a) and rat IGFBP-4 has been shown to be glycosylated (Ceda *et al.*, 1991). In agreement with the rat data, results from collaborators (Walton, P. E. and Grant, P. A., CRC for Tissue Growth and Repair, Adelaide, South Australia) have shown oIGFBP-4 to exist in plasma as two different molecular weight forms; 29 and 24 kDa, that differ due to the presence of N-

linked sugars on the former (Carr *et al.*, 1994a). Thus, the potential N-linked glycosylation site on oIGFBP-4 is utilised *in vivo*. The suggested hydrophilic nature of oIGFBP-4 is not surprising considering its role in the circulation. Computer structural predictions, based on linear protein sequence, suggest N-terminal β-sheet structures with the remainder of the protein comprised of several α-helices. This is in agreement with recently presented data generated by computer modelling of human IGFBP-4 using 3-D analysis and energy minimisation (Landale *et al.*, 1994).

Northern analysis of oIGFBP-4 mRNA was performed to characterise mRNA size and distribution in the sheep. IGFBP-4 mRNA transcripts of approximately 2.6, 2.1 and 1.8 kb were present at different levels in various tissues from the adult and fetus at both early (0.3 gestation) and late (near term) gestational ages. In the adult ewe, IGFBP-4 mRNA was highest in the kidney ≥ liver > lung and low in the heart. This is similar to the tissue distribution of IGFBP-4 mRNA in the adult rat, which is the only other published analysis of IGFBP-4 mRNA in adult tissues, although IGFBP-4 mRNA levels were markedly higher in the rat liver than any other tissue examined (Shimasaki *et al.*, 1990a). The size of the mRNA transcripts for oIGFBP-4 (2.6 kb) is comparable to those reported for other species (Shimasaki *et al.*, 1990a; Ceda *et al.*, 1991; Moser *et al.*, 1992). In the fetus, the wide distribution of IGFBP-4 mRNA throughout gestation contrasts to that shown for IGFBP-2, for which the mRNA becomes restricted to primarily the liver and kidney in late gestation. This is suggestive of a more ubiquitous and fundamental role of IGFBP-4 in tissue growth and development than that of IGFBP-2. Comparison of IGFBP-4 mRNA levels within different tissues and between the adult and fetus, particularly in the heart, suggest IGFBP-4 mRNA levels are regulated in a tissue and developmental specific manner. These observations are the first reports of ontogenic changes in IGFBP-4 mRNA in the fetus and were subsequently investigated further in Chapter 5.

Northern analysis also suggested the presence of multiple transcripts for oIGFBP-4. Sheikh *et al.* (1993) have observed multiple transcripts for human IGFBP-4 in breast carcinoma cells and suggested the smaller transcripts to represent cross hybridisation with 18s rRNA. Several lines of evidence suggest that this may not be the case for oIGFBP-4:

- multiple transcripts have been reported by independent investigators for IGFBP-4 in rat neuroblastoma and human fibroblast cell lines (Ceda *et al.*, 1991; Camacho-Hubner *et al.*, 1992)
- there are two extra bands for oIGFBP-4 while for 18S rRNA there should only be one
- the bands observed are not the same size as 18S rRNA
- the binding is specific and not removed at stringencies up to 68^{o} C in 0.1 X SSC
- the levels of the smaller bands are related to IGFBP-4 mRNA levels and not RNA loading.

The relevance of multiple transcripts for IGFBP-4 is unknown, but may be due to alternate promoter usage or splicing, as in the case for IGF-I and IGF-II (De Pagter-Holthuizen *et al.*, 1987; Rotwein, 1991; Jansen *et al.*, 1992). The multiple transcripts, conserved 3' sequences and potential for protein glycosylation, indicated in this chapter, suggest that the regulation of oIGFBP-4 may be intricate with potential control at the level of protein glycosylation, translation, mRNA stability or transcription. Another point of control, not addressed in this study, is via the IGFBP-4 specific proteases (Fowlkes and Freemark, 1992a; Conover *et al.*, 1993a; Kanzaki *et al.*, 1994) (1.2.5.2).

The physiological functions and regulation of IGFBP-4 are currently under investigation. The availability of DNA probes for oIGFBP-4 will enable examination of the control of this protein in the sheep at the level of the gene.

CHAPTER 4. GENERATION OF SPECIFIC OVINE IGFBP-4 ANTIBODIES

4.1 INTRODUCTION

Western ligand blotting is a useful technique for parallel assessment of different IGFBPs in the same samples and relative quantitation of IGFBP levels can be performed through the careful use of controls. However, the Western ligand blotting technique has several disadvantages:

(1) Western ligand blotting does not detect all IGFBPs (Ocrant *et al.*, 1992).

(2) the method is time consuming with large numbers of samples.

(3) measurement of IGFBPs by Western ligand blot analysis is not as precise as many other techniques due to gel loading and transfer variability.

(4) detection by Western ligand blot is based upon IGF binding affinity and may be affected by post-translational mechanisms that may alter the affinity of IGFBPs for IGF, without changes in IGFBP protein levels (see 1.2.5).

Therefore, although measurements of IGFBPs by Western ligand blotting is satisfactory to allow relative comparisons of the amount of IGF binding activity present under different conditions or in different biological fluids, it is not amenable to routine quantitative measurements of IGFBP concentrations in a large number of samples. Thus, to further our understanding of the roles and regulation of IGFBPs at the protein level, sensitive and specific protein assays must be developed.

Previous work by P. A. Grant and P. E. Walton, CRC for Tissue Growth and Repair, Adelaide, South Australia, has established a system for measurement of oIGFBP-3 by a specific radioimmunoassay (RIA). However, in generating an antibody against purified native oIGFBP-4, with which to develop a similar oIGFBP-4 RIA, the investigators produced an antibody with marked cross-reactivity to oIGFBP-2. A commercially available antibody to human IGFBP-4, (Upstate Biotechnology Incorporated, Lake Placid, NY, USA, anti IGFBP-4 polyclonal, Cat # 06-109), is also reported to possess up to 50 % cross-reactivity with IGFBP-2, suggesting the presence of a common epitope on these two proteins. In view of this lack of specific IGFBP-4

antibodies, the aim of this study was to develop antibodies using specific peptides directed against regions of oIGFBP-4 with low homology to oIGFBP-2, to avoid these problems of antibody cross-reactivity.

Traditionally synthetic peptides and small polypeptides are not effective antigens and must be conjugated to larger carrier proteins, such as albumin, to enhance their immunogenic potential (Muller, 1988). However, a more novel approach to peptide immunisations through the use of a hybrid bacteriophage display system has been chosen for this study (Greenwood, *et al.*, 1991). The hybrid bacteriophage display system was developed and kindly provided to us by Professor R. N. Perham and Dr. A. Willis, Department of Biochemistry, University of Cambridge, UK. The system involves introducing DNA encoding the peptide of interest into a plasmid containing the gene for the major coat protein (gene VIII) of the filamentous bacteriophage, fd (Rasched and Oberer, 1986), under control of an inducible promoter. Cells containing this plasmid are induced to produce recombinant peptide-coat protein fusion molecules and at the same time are super-infected with wild type fd. The peptide-coat protein fusion molecules are packaged into bacteriophage particles, along with wild type coat protein, translated from the infecting wild type fd. These hybrid bacteriophage particles, containing both wild type and peptide-coat protein fusion molecules are isolated and used directly as antigens.

Using this system, an antibody specific for oIGFBP-4 has been generated. This will be valuable for the development of a specific oIGFBP-4 assay and may also be useful in studies investigating IGFBP-4 structural or functional relationships.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Cesium chloride (molecular biology grade) was obtained from Boehringer Mannheim, Germany. Ammonium sulfate (ultra pure) and disuccinyl suberate (DSS) were obtained from ICN Biomedicals, Inc. Cleveland, OH, USA. Tricine was obtained from Aldrich Chemical Company, Inc. Milwaukee, WIS, USA. 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), Coomassie Brilliant Blue R-250, sodium azide and

incomplete and complete Freunds adjuvant were obtained from Sigma Chemical Company, St. Louis, MO, USA. Na_2CO_3, $CuSO_4$, KI and silver nitrate were obtained from BDH Chemicals Australia, Pty. Ltd., Kilsyth, Vic. Australia. Methanol was obtained from Merck Pty Ltd., Kilsyth, Vic., Australia. Dimethyl sulphoxide (DMSO), citric acid and NH_4OH were obtained from AJAX, Sydney, Australia. Glutaraldehyde (25 %, Electron microscope grade) was obtained from TAAB Laboratories Equipment Limited, Reading, UK. Polycarbonate ultracentrifuge tubes (10 ml) were purchased from Beckman Instruments Inc. Palo Alto, CA, USA. Polyvinylidene difluoride (PVDF) membranes (0.2 µm) were purchased from Biorad Laboratories Inc., Herates, CA, USA. Sephadex G-100 was from Pharmacia, Uppsala, Sweden. Dialysis tubing (molecular weight cut off 3,500 Da, Spectra/Phor^6 membrane) was purchased from Spectrum Medical Industries Inc. CA, USA. Anti-rabbit IgG was obtained from Silenus Laboratories, Hawthorn, Australia and rabbit IgG was purchased from Sigma Chemical Company, St. Louis, MO, USA. Anti-rabbit IGFBP-4 polyclonal antibody was kindly donated by Mrs. P. A. Grant and Dr. P. E. Walton, CRC for Tissue Growth and Repair, Adelaide, South Australia.

4.2.1.1 Solutions

PBS	50 mM sodium phosphate	pH 7.0
(Phosphate-buffered saline)	0.15 M NaCl	
Microbiuret Reagent	0.01 M $CuSO_4$	
(adapted from Mokrasch and	1.4 % (v/v) NH_4OH	
McGilvery, 1956)	4.4 M NaOH	
	6 mM KI	

4.2.1.2 DNA Oligomers

A

```
          P   C   S   A   H   D   R   R   C   L   Q
2547 5' CCG TGC TCC GCT CAC GAC CGT CGT TGC CTG CAG 3'
2548 3' GGC ACG AGG CGA GTG CTG GCA GCA ACG GAC GTC 5'
```

B

```
          V   I   G   A   P   R   E   E   V   R   P
2549 5' GTT ATC GGT GCT CCG CGT GAA GAA GTT CGT CCG 3'
2545 3' CAA TAG CCA CGA GGC GCA CTT CTT CAA GCA GGC 5'
```

DNA Sequencing oligomers

1789 : 18 mer, binds to the trc promoter in the vector, pKfdH

5' GGCAAATATTCTGAAATG 3'

1714 : 21 mer, binds to the 5S rRNA transcription termination signal in pKfdH

5' TCA GGC TGA AAA TCT TCT CTC 3'

4.2.1.3 E. coli Strains

JM101 : *supEthi*Δ(*lac-proAB*)

F'[*traD*36*proAB*$^{+}$*lacI*^{q}*lacZ*ΔM15]

JM109 : *recA*1*supE*44*endA*1*hsd*R17*gyrA*96*relA*1*thi*Δ(*lac-proAB*)

F'[*traD*36*proAB*$^{+}$*lacI*^{q}*lacZ*ΔM15]

(Sambrook *et al.*, 1989)

4.2.1.4 Biological Fluids

Ovine plasma was obtained by jugular venipuncture of three non-pregnant ewes. Human plasma was donated by Dr. S. E. Gargosky and was obtained from a single non-pregnant female. Rat plasma was donated by Ms. S. E. Bastian, obtained from a pool of non-pregnant female rats. Fetal bovine serum was purchased from Flow Laboratories,

North Ryde, NSW, Australia. Chicken plasma was donated by Dr. B. E. Forbes and Ms. K. Niedzielski and was obtained from broiler chickens at slaughter. Porcine plasma was donated by Ms. P. A. Grant, obtained from adult pigs. L6 rat myoblast and HE39L human lung fibroblast culture medium was collected under serum free conditions and concentrated, 10 fold, by centrifugation with Centricon-10 concentrators (Amicon, INC, Beverly, MA, USA), and kindly donated by Ms. B. Magee and Mr. G. Shooter.

4.2.2 CLONING OF DNA OLIGOMERS

4.2.2.1 Preparation of oligomers and plasmid, pkfdH

Complementary DNA oligomers, optimised for codon usage in *E. coli*, were synthesised to two regions of IGFBP-4 as shown in Figure 4.1, and purified by gel electrophoresis. Oligomers (20 µg) were heat denatured in 50 % deionised formamide and electrophoresed on a 0.5 mm, 7 M Urea, 1 X TBE, 10 % polyacrylamide gel (w/v, 25:1, polyacrylamide : bis-acrylamide) at 15 mA. DNA bands were visualised and excised by shadowing under ultra violet (UV) light onto cellulose/fluor 300 polyethyleneimine impregnated chromatography paper (Machery-Nagel and Co., Germany). Oligomers were eluted into 0.5 M NH_4OAc, 10 mM MgOAc, 1 mM EDTA, 0.1 % (w/v) SDS, overnight at 37° C and precipitated with two volumes of ethanol at -20° C. DNA recovery and concentration was determined by spectral analysis (2.2.4). Approximately 5 µg of each oligomer were heat denatured and phosphorylated as described in section 2.2.1.2. The plasmid pkfdH (Figure 4.2) was digested with 1 U of *Hpa*I in 1 x Superduper buffer (2.1.3) at 37° C for two hours. The linearised vector was purified by GENECLEAN, dephosphorylated with calf intestinal alkaline phosphatase and re-purified on a Sepharose CL-6B spin mini-column (2.2.1). Approximately 2.5 µg of each complementary oligomer were annealed in 0.5 M NaCl by denaturing at 95° C, five minutes then slow cooling in a 70° C heating block, placed at room temperature. Once the temperature of the heating block had fallen to room temperature, the annealing reaction was diluted 1/20 in 1 x TE and annealed oligomers immediately added to the ligation reaction.

4.2.2.2 Ligation, transformation and screening of transformants

Approximately 125 ng of annealed oligomers (ie 1/20th of the diluted annealing reaction) were ligated to an estimated equal amount of dephosphorylated, *Hpa*I digested pkfdH (2.2.1.2). Competent *E. coli* JM109, were prepared and transformed with 1/3 of the ligation mixture (2.2.1.1). Twelve colonies from each ligation were selected and plasmid DNA extracted (2.2.1.3). One third of the mini-prep DNA was denatured, desalted on a Sepharose CL-6B spin column and sequenced with specific DNA oligomers to the promoter (1789) and transcription termination region (1714) of the vector pKfdH (2.2.1.5). Clones with the DNA oligomers inserted as single copies in the correct orientation were selected for production of hybrid bacteriophage.

4.2.3 PRODUCTION AND PURIFICATION OF HYBRID BACTERIOPHAGE

Plasmid DNA was transformed into competent JM101 cells and maintained on MinA medium to retain the F pilis for subsequent bacteriophage infection. A single colony was inoculated into 5 ml MinA + ampicillin (50 µg /ml) and grown overnight at 37° C. Two ml of overnight culture was added to 500 ml 2YT + ampicillin (50 µg/ml), mixed, and 5 ml immediately subcultured and infected with approximately $1x10^8$ pfu of wild type fd. Both cultures (5 ml infected and 500 ml uninfected) were grown at 37° C for two hours or until the A_{600} nm of the 500 ml culture was approximately 0.2 to 0.3 OD units. The 500 ml culture was induced with IPTG (0.1 mM) and grown for 20 minutes at 37° C. The infected 5 ml culture was added to the induced 500 ml culture and grown overnight at 37° C (final A_{600} nm approximately 2.5 to 3.5 OD units). Cells were removed by centrifugation at 10,000 x g for 10 minutes and the supernatant further clarified by centrifugation, twice, as above. Bacteriophage particles were precipitated from the supernatant by incubation at room temperature for 30 minutes in 3 % (w/v) PEG 6000, 0.5 M NaCl and pelleted by centrifugation, 10,000 x g for 20 minutes. Pellets were drained and resuspended in 5 ml TE, pH 7.5. Further TE was added, dropwise, to a weight of 10 g, then cesium chloride to 42 % (w/w), in a 10 ml polycarbonate ultracentrifuge tube. The solution was centrifuged at 145,000 x g, 24 hours at 20° C. The opaque

bacteriophage band was removed by carefully piercing the tube below the bacteriophage band with a hot pin and collecting the eluate, dropwise (1 to 2 ml). The bacteriophage preparation was dialysed against TE overnight at 4° C.

4.2.4 ANALYSIS OF HYBRID BACTERIOPHAGE

4.2.4.1 Quantitation of bacteriophage by microbiuret assay

The concentrations of bacteriophage protein preparations were quantitated using the microbiuret assay, adapted from Mokrasch and McGilvery, (1956). Bacteriophage preparation (20 µl) was incubated with an equal volume of 1 M NaOH at room temperature for one hour, diluted to 200 µl with TE then 800 µl of microbiuret reagent added. Samples were mixed and A_{300} nm determined. Absorbances of BSA standards (0 to 100 µg/ml) were determined in parallel to construct a standard curve from which the concentration of protein in the bacteriophage samples was determined.

4.2.4.2 Purity of bacteriophage by Tricine SDS-PAGE

The purity of bacteriophage preparations were visually assessed by Tricine SDS-PAGE, using a method adapted from Shagger and von Jagow, (1987). Approximately 2 to 4 µg of bacteriophage was reduced and denatured by heating for five minutes at 100° C in 0.1 M DTT, 2 % (w/v) SDS, 10 % (v/v) glycerol, 15 mM Tris, pH 7.5. Protein was electrophoresed on a 5 % (w/v, 25 : 1, acrylamide : bis-acrylamide), 0.75 M Tris, pH 8.3, stacking gel and 16.5 % (w/v, 25 : 1, acrylamide : bis-acrylamide) separating gel in 0.1 M Tricine, 0.1 M Tris, 0.1 % (w/v) SDS at 100 V, constant voltage.

Proteins were detected by Coomassie Brilliant Blue R-250 staining. Briefly, gels were stained in 0.1 % (w/v) Coomassie Brilliant Blue R-250, 50 % (v/v) ethanol, 10 % (v/v) acetic acid at room temperature for 60 minutes then destained in several changes of 10 % (v/v) acetic acid, 5 % (v/v) ethanol until the desired level of destaining was achieved. For detection of lower quantities of hybrid bacteriophage particles, gels were silver stained, as described by Morrisey, (1981). Gels were pre-fixed at room temperature in 50 % (v/v) methanol, 10 % (v/v) acetic acid for 30 minutes, followed by 5 % (v/v) methanol,

7 % (v/v) acetic acid for a further 30 minutes. Gels were then fixed in 10 % glutaraldehyde for 30 minutes and rinsed in several changes of water over two hours. Proteins were reduced with 5 µg/ml DTT for 30 minutes and treated with 0.1 % (w/v) silver nitrate for a further 30 minutes. Gels were rinsed with water then developer (0.05 % [v/v] of 37 % formaldehyde solution in 3 % [w/v] Na_2CO_3), followed by soaking in developer until the required intensity of staining was obtained. The reaction was stopped with 1/20 th volume 2.3 M citric acid and rinsed with water. Coomassie Brilliant Blue R-250 stained or silver stained gels were soaked in 30 % (v/v) ethanol, 5 % (v/v) glycerol for at least one hour and preserved by air drying between cellophane sheets as described by Michaels and Ford, (1991).

4.2.4.3 Protein sequencing

The protein sequence of the hybrid bacteriophage major coat protein was determined by electroblotting onto PVDF membranes as described by Matsudaira, (1988). Approximately 500 ng (70 pmoles) of bacteriophage preparation was electrophoresed on Tricine SDS-PAGE, as described above. Proteins were then transferred to 0.22 µM PVDF membranes in 10 mM CAPS, 10 % (v/v) methanol, pH 11.0, at 300 mA for 30 minutes. The membranes were washed for five minutes in water, stained for five minutes in 0.5 % (w/v) Coomassie Brilliant Blue R-250, 50 % (v/v) methanol, 5 % (v/v) acetic acid, and destained for 10 minutes in 50 % (v/v) methanol, 10 % (v/v) acetic acid. The stained protein bands (wild type and hybrid coat proteins) were excised and stored in a tube at -20° C for sequencing. Protein sequence analysis was performed by Ms D. Turner using an Applied Biosystems 470 A gas phase sequencer, as described by Hunkapiller *et al.*, (1983).

4.2.5 GENERATION OF ANTIBODIES

4.2.5.1 Immunisations

Antibodies were raised by immunisation of rabbits. Two New Zealand White rabbits for each peptide region selected (ie. four rabbits, total) were immunised by subcutaneous injection into 8 to 10 sites across the back of either:

(i) 100 µg bacteriophage + complete Freunds adjuvant or

(ii) 200 µg bacteriophage alone.

Animals were boosted at monthly intervals with 50 µg of bacteriophage in incomplete Freunds adjuvant, with blood being collected, 10 to 12 days after boosting, by ear vein bleed. After a period of eight months a terminal bleed was taken, under anaesthesia, and animals sacrificed. Blood samples were collected and allowed to clot overnight at 4° C. Serum was obtained by centrifugation at 1,500 x g, 20 minutes, dispensed and stored at -15° C. All boosts and bleeds were carried out by Mr Brian Miller at the University of Adelaide Central Animal House. Initial immunisations were performed with the aid of Mrs. P. A. Grant, CRC for Tissue Growth and Repair, Adelaide, South Australia.

4.2.5.2 Ammonium sulphate precipitation of IgGs

A portion of the serum obtained from the terminal bleed was further processed by ammonium sulphate precipitation of IgGs. Serum was clarified by centrifugation, 18,000 x g, 15 minutes at 4° C. The supernatant was taken and ammonium sulphate (30 % [w/v]) added slowly with gentle swirling. The solution was then incubated at 4° C for 30 minutes with gentle rocking. Precipitated proteins were pelleted by centrifugation, 10 minutes, 12,000 x g at 4° C, resuspended in 50 mM phosphate-buffered saline (PBS) and dialysed overnight at 4° C against 50 mM PBS + 0.02 % (w/v) sodium azide. The A_{280} nm was measured and protein concentrations determined based on A_{280} nm of 1 mg/ml protein solution = 1.4. The IgG fractions were dispensed and stored at -15° C.

4.2.6 SCREENING ANTISERA

4.2.6.1 Western immunoblotting of Antisera

Antisera were analysed for the presence of fd antibodies after the second bleed. Approximately 1 µg of bacteriophage preparation was subjected to Tricine SDS-PAGE as described in 4.2.4.2. Proteins were transferred to 0.22 µm Nitrocellulose filters and probed with antisera at a dilution of 1/2000, as described in section 2.2.3.2. Additionally, ovine

plasma samples (2 µl) were screened by Western immunoblotting, with antisera at dilutions up to 1/250. Western immunoblotting of plasma failed to show the presence of IGFBP-4 specific antibodies. Antisera were then screened for IGFBP-4 antibodies by radioimmunoprecipitation of pure oIGFBP-4 cross-linked to ^{125}I-IGF or by immunoprecipitation of native IGFBP-4 from ovine plasma, as described below.

4.2.6.2 Cross-linking ^{125}I-IGF to ovine IGFBP-4

IGF-I and IGF-II were iodinated as described in section 2.2.3.3. Approximately 1 µg of oIGFBP-4 was equilibrated with 0.1 µg of ^{125}I-IGF-I in 1 ml of 50 mM sodium phosphate, pH 6.5 at room temperature for two hours. Ten microliters of 0.9 % (w/v) DSS in DMSO was added to the IGF/IGFBP-4 complexes, and the reaction incubated for 30 minutes at room temperature. The cross-linking reaction was stopped by the addition of 50 µl 1 M Tris, pH 8.0 and 5 µl kept aside as a measurement of total counts. Cross-linked ^{125}I-IGF was separated from free ^{125}I-IGF, high molecular weight cross-linked products and ^{125}I by Sephadex G-100 column chromatography, on a 40 x 1 cm column. The column was run under gravity, in 0.5 M acetic acid, pH 3.0, 0.1 M NaCl, 0.25 % (w/v) BSA at a flow rate of 0.16 ml/min and 120 fractions, each of two minutes, were collected. Five microliters from each fraction was neutralised with an equal volume of 1 M Tris, pH 9.0, counted (LKB Wallac 1261 Multigamma Counter, LKB, Turku, Finland), then subjected to SDS-PAGE. PAGE gels were transferred to Whatman 3MM paper, dried under vacuum and autoradiographed for three days at -80^{o} C. Fractions containing primarily ^{125}I-IGF/IGFBP-4 complexes with little ^{125}I-IGF were pooled and stored at -20^{o} C for use in subsequent radioimmunoprecipitation reactions.

4.2.6.3 Radioimmunoprecipitation of ^{125}I-IGF/IGFBP-4

Antisera were diluted as required in 50 mM PBS and 50 µl added to 200 µl of RIA buffer (50 mM sodium phosphate, pH 7, 150 mM NaCl, 0.25 % [w/v] BSA, 0.05 % [v/v] Tween-20, 0.02 % [w/v] sodium azide). ^{125}I-IGF/IGFBP-4 cross-linked tracer (50 µl, approximately 10,000 cpm) was added to each tube, which were mixed and incubated

overnight at 4° C. Anti-rabbit IgG (50 µl, diluted 1/20) and 10 µl of 1/200 diluted rabbit IgG were added to each tube which were then mixed and incubated at 4° C for 30 minutes. Complexes were precipitated by the addition of 1 ml 6 % (w/v) PEG 6,000, 150 mM NaCl and pelleted by centrifugation for 20 minutes at 8,000 x g at 4° C. The supernatant was aspirated and pellets counted.

4.2.6.4 Immunoprecipitation of IGFBP-4 from ovine plasma

Immunoprecipitation of IGFBP-4 from plasma was carried out as described above for radioimmunoprecipitation, with the exception that 20 µl of ewe, bovine, porcine or rat plasma, was diluted to 50 µl with RIA buffer and substituted for cross-linked tracer. Additionally, 50 µl human or chicken plasma, L6 rat myoblast or HE39L human lung fibroblast culture medium (serum free, 10 x concentrated) were immunoprecipitated. Antisera were used at a final dilution of 1/60. Aspirated pellets were resuspended in 20 µl 50 mM PBS and half of each pellet subjected to SDS-PAGE and Western ligand blotting (2.2.3.1).

4.3 RESULTS

4.3.1 GENERATION OF DNA CLONES

The peptide regions of oIGFBP-4 selected for generating antibodies are shown in Figure 4.1. The positions of these peptide regions within the IGFBP-4 protein are shown in Chapter 3, Figure 3.5. The hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982) and antigenicity profiles (Welling *et al.*, 1985; Jameson and Wolf, 1988), for human, rat and oIGFBP-4 that were considered in selecting these peptide regions, are shown in Appendix I. Profiles for bovine IGFBP-4 are also shown. Complementary DNA oligomers for peptides A and B, with codon usage optimised for *E.coli*, were annealed and cloned into the vector pkfdH. Details of this plasmid are illustrated in Figure 4.2, adapted from the publication of Greenwood *et al.* (1991). The vector utilises a unique *Hpa*I restriction endonuclease site as the DNA insertion site, which was engineered between the signal sequence and mature N-terminus of the gene VIII, major coat protein, of the

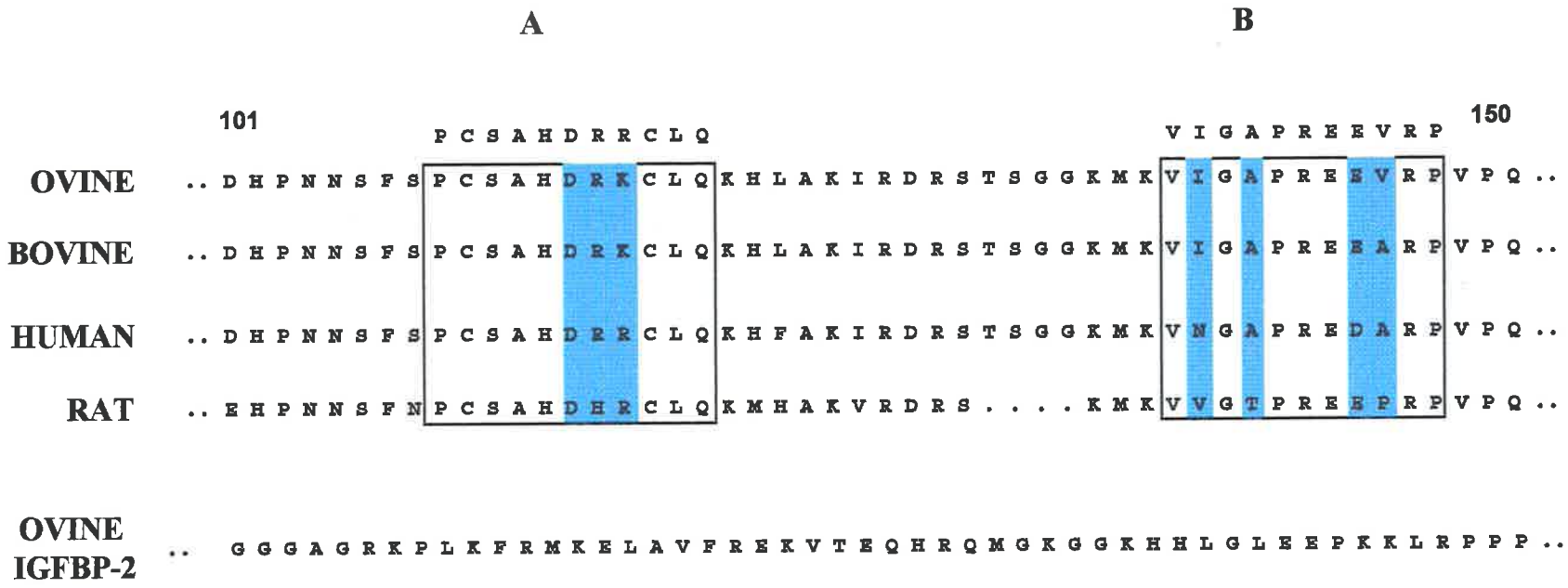


FIGURE 4.1 PEPTIDE REGIONS OF IGFBP-4 USED FOR IMMUNISATIONS. Known sequences for human and rat IGFBP-4 and partial sequence for ovine IGFBP-4 were compared and subjected to computer determinations of antigenicity, hydrophilicity and surface probability (Appendix I). The N-terminal 132 amino acids of ovine IGFBP-4 and sequence for bovine IGFBP-4 were not available at the time of analysis. Peptide regions A and B (boxed) were selected as peptide immunogens, the sequences of which are shown above the boxes. The sequence for ovine IGFBP-2 over a similar region of the protein is indicated. Residues highlighted in blue indicate amino acid positions in peptides A and B not conserved across all known sequences for IGFBP-4.

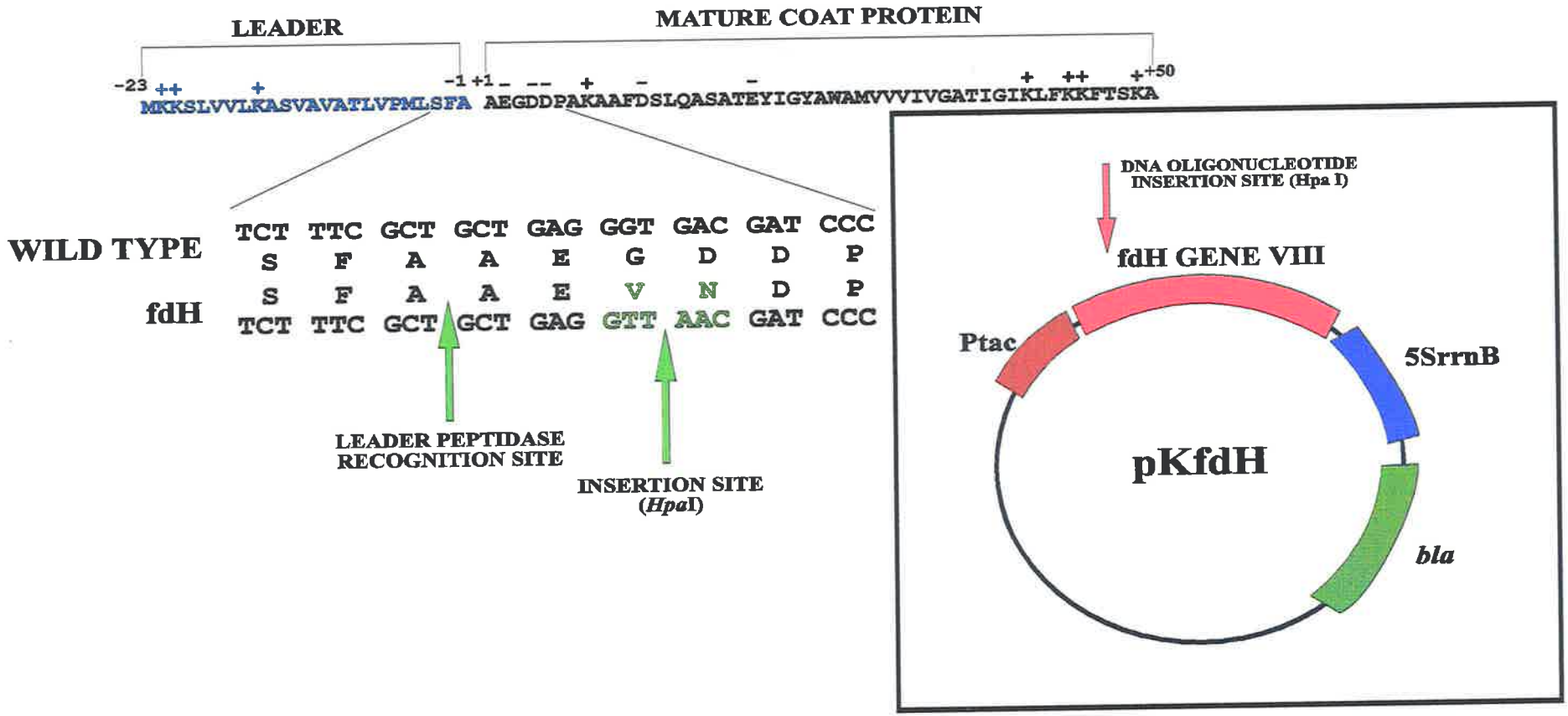


FIGURE 4.2 THE EXPRESSION VECTOR pKfdH. The expression vector pKfdH was provided by Professor R. N. Perham and contains the coding region for the major coat protein (gene VIII) of the bacteriophage, fd, under control of an IPTG inducible promoter. A *Hpa*I restriction site has been engineered between the leader peptide and the N-terminus of the mature protein (creating the mutant fdH gene VIII), into which DNA encoding the peptides of interest can be inserted. The vector also contains a signal for termination of transcription (5S*rrnB*) and the *bla* gene for ampicillin resistance. Adapted from Greenwood *et al.* (1991).

bacteriophage fd. The peptide-coat protein fusion protein is under control of the IPTG-inducible, ptac promoter (a lac, trc hybrid promoter). The vector also has the 5S rRNA termination of transcription signal (5S*rrnB*). Possible recombinant clones were screened by DNA sequencing for the insertion of the DNA oligomers. Clones were identified with the DNA oligomers inserted in the correct or reverse orientation and as oligomer concatamers, as is illustrated in Figure 4.3. Two clones, A and B, with the DNA oligomers inserted as single copies in the correct orientation, were used for subsequent production of hybrid bacteriophage.

4.3.2 PURIFICATION AND ANALYSIS OF HYBRID BACTERIOPHAGE

Clones A and B (Figure 4.3) were transformed into *E. coli* JM101. Cells were induced to produce fusion peptide-coat protein molecules and co-infected with wild type fd, resulting in the production of hybrid bacteriophage particles. Bacteriophage particles were purified and analysed by Tricine SDS-PAGE (Figure 4.4). Comparison of bacteriophage isolated from induced cells containing clone A or B with bacteriophage isolated from induced cells containing the vector pkfdH, indicates the presence of an additional minor band of slightly higher molecular weight than the normal gene VIII, coat protein product (Figure 4.4). Bacteriophage coat protein molecules (both bands) were transferred to PVDF membranes and protein sequenced. Results indicate the presence of both fusion peptide-coat protein and wild type coat protein molecules, as expected (Figure 4.5).

4.3.3 IMMUNISATION AND SCREENING OF ANTISERA

Rabbits were immunised with purified hybrid bacteriophage and resulting antisera screened for the presence of antibodies directed against fd. Western immunoblot of bacteriophage fd preparations showed the presence of antibodies in all rabbits by the second boost. Immunoblots of antisera Ai and Bii against fd are shown (Figure 4.6). Results indicate the presence of immunoreactivity against bacteriophage proteins and an extra band, representing the fusion-peptide gene VIII product, in fd + A and fd + B.

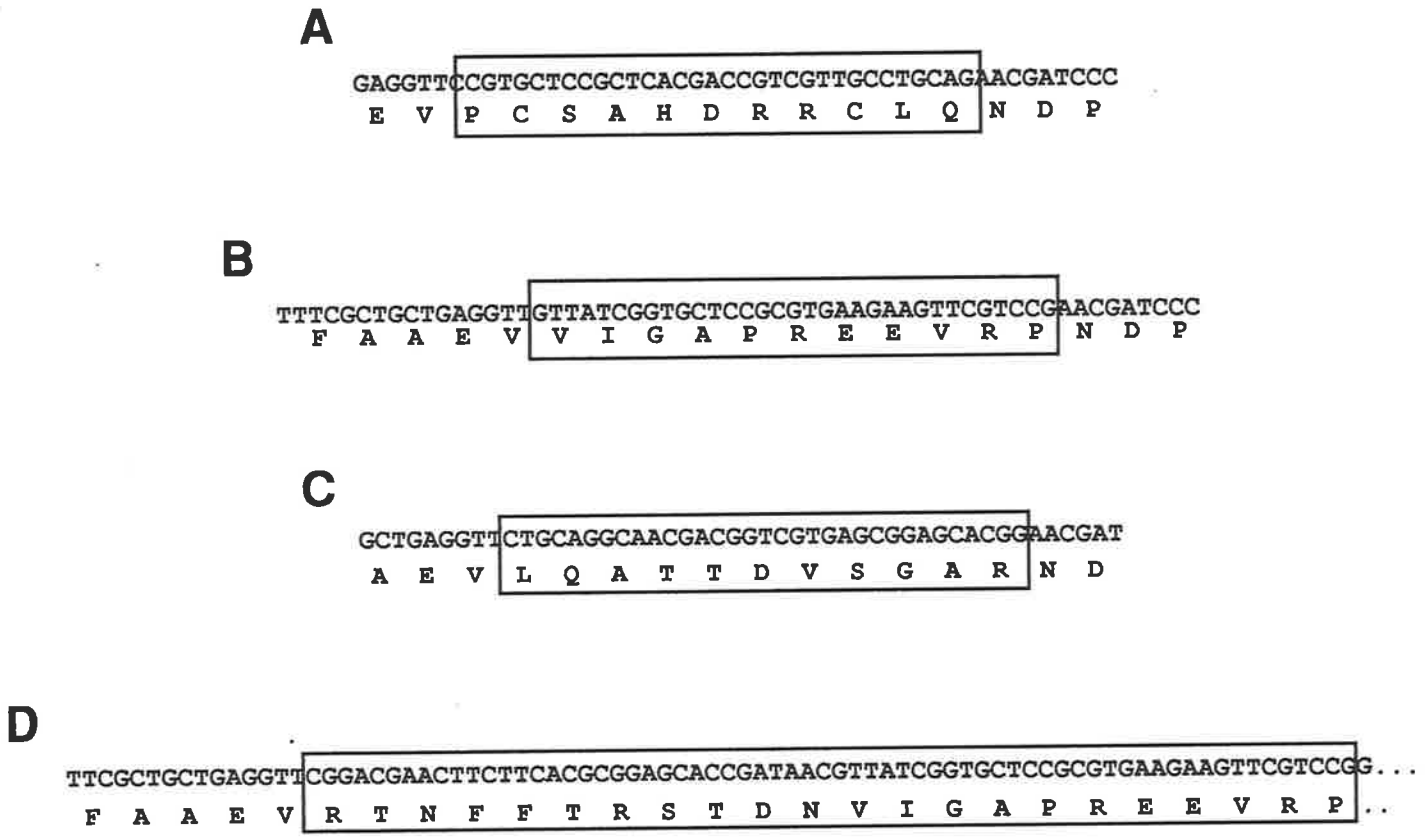


FIGURE 4.3. DNA SEQUENCE OF CLONES. Possible recombinant DNA clones were screened by sequencing which identified several types of clones (A to D). The DNA sequence over the *Hpa*I insertion site for four different clones is shown. Some clones contained oligomer concatamers, both in the correct or reverse orientation (C and D). DNA inserts are boxed. Clones A and B, containing single copy oligomers inserted at the *Hpa*I site in the correct orientation, were selected for hybrid bacteriophage production.

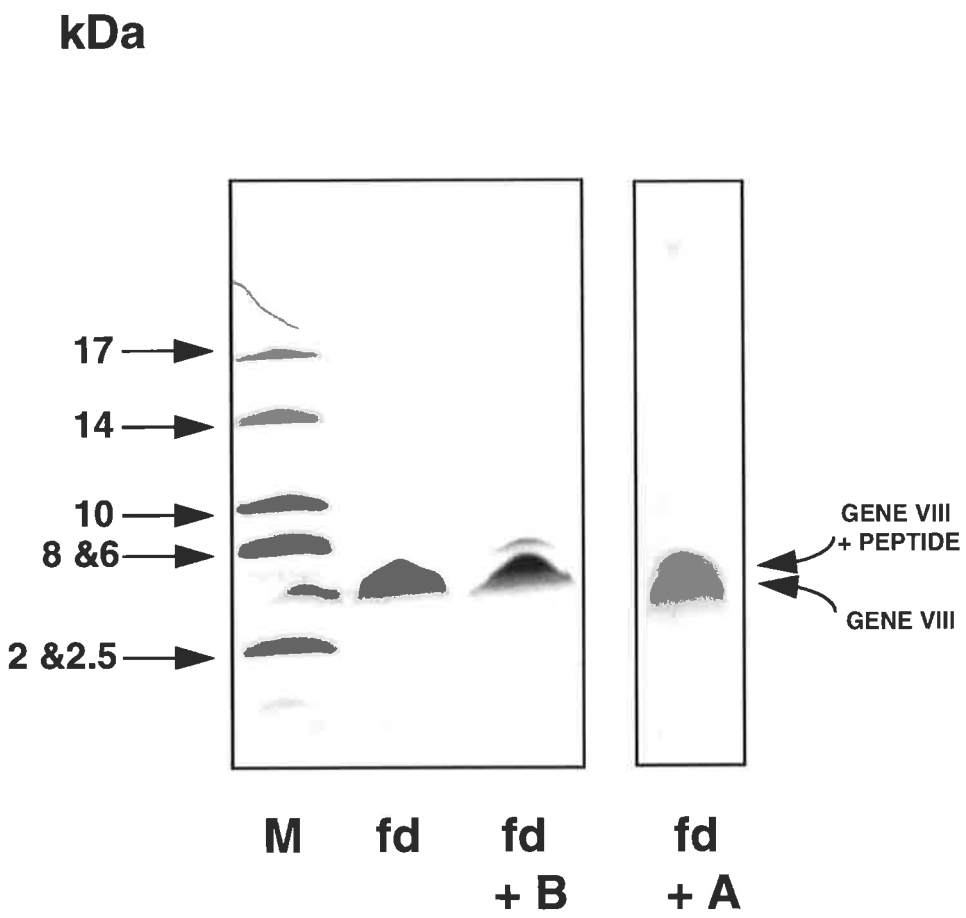


FIGURE 4.4 TRICINE SDS-PAGE ANALYSIS OF HYBRID BACTERIOPHAGE PREPARATIONS. Hybrid bacteriophage were isolated from cells containing the vector pKfdH (fd), clone A (fd + A) and clone B (fd + B), as described in section 4.2.3. Bacteriophage particles were subjected to Tricine SDS-PAGE and Coomassie Blue-R250 staining (4.2.4.2). Protein bands representing wild type major coat protein, (gene VIII), and the peptide-gene VIII fusion protein are indicated. M = molecular weight markers. Wild type gene VIII protein is approximately 5.2 kDa.

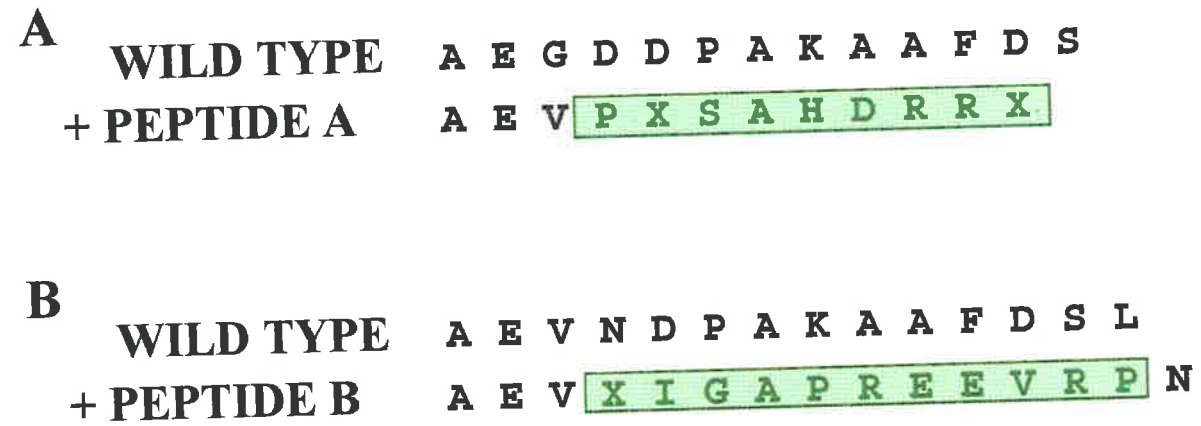


FIGURE 4.5 PROTEIN SEQUENCING OF GENE VIII PROTEIN FROM HYBRID BACTERIOPHAGE PREPARATIONS. Hybrid bacteriophage were prepared, from cells containing clone A or clone B, as described in section 4.2.3. Bacteriophage particles were subjected to reducing SDS-PAGE, electroblotted onto PVDF membranes, gene VIII protein bands excised and protein sequenced (4.2.4.3). Both wild type and peptide fusion proteins were detected. Boxed regions indicate inserted peptides A and B. X=amino acid not determined. In peptide A, X = cysteine residues. In peptide B, X = V (Val) and may not have been detected due to lag from the preceeding V residue during protein sequencing.

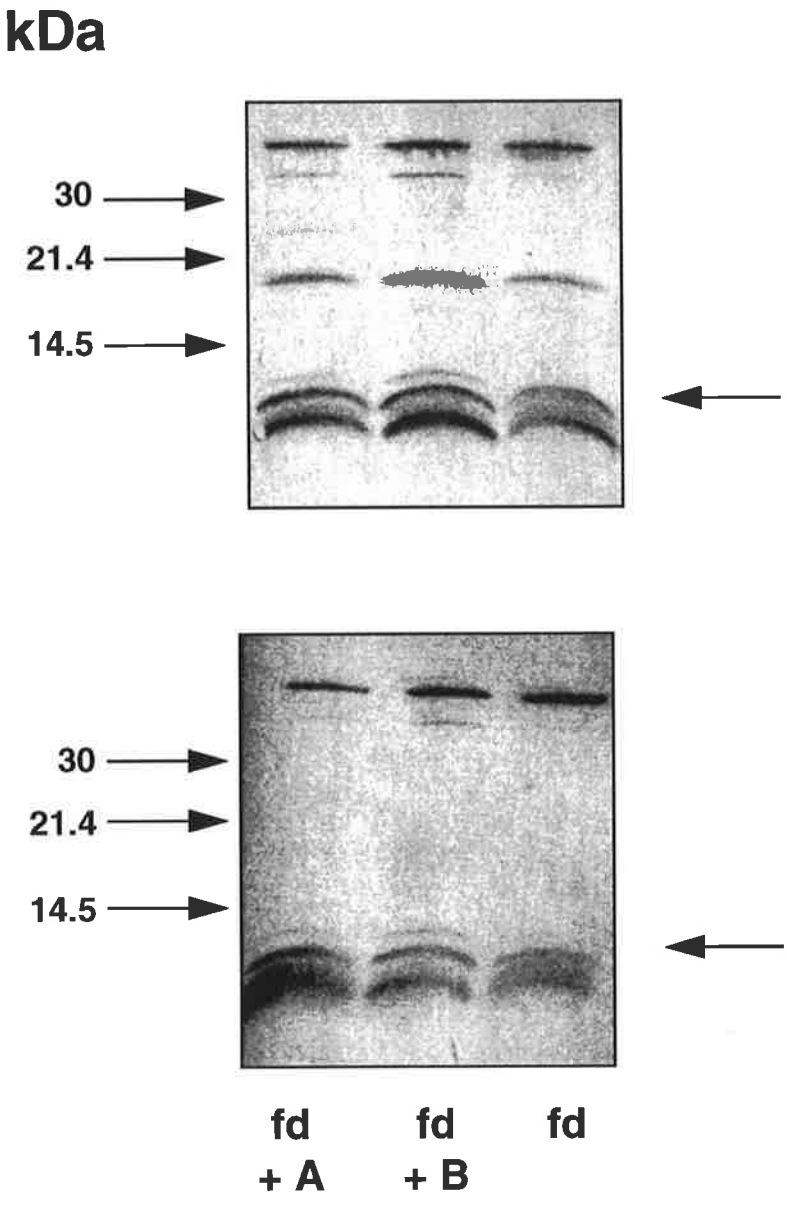


FIGURE 4.6 WESTERN IMMUNOBLOT OF ANTIBODIES AGAINST fd. Antisera obtained from the second bleed were screened for immunoreactivity against bacteriophage preparations from cells containing the vector pKfdH (fd), clone A (fd + A) and clone B (fd + B). Approximately 1 µg of bacteriophage protein was electrophoresed, transferred to nitrocellulose and immunoblotted with antisera at 1/2000 dilution, as described in 4.2.6.1. Immunoreactive species were detected, indicating sucessful generation of antibodies to fd. The arrow indicates the additional immunoreactive species observed in fd + A or B. Results of antisera from rabbits Ai (upper panel) and Bii (lower panel) are shown. Antisera from rabbits Aii and Bi gave similar results to those shown for Ai.

Figure 4.6 also shows the presence of multiple immunoreactive species probably due to the production of antibodies against other minor bacteriophage coat proteins (gene III, gene 6, 7 or gene 9) and contaminating *E. coli* proteins in the bacteriophage preparation. Variability of the immune responses between rabbits is illustrated by comparison of the immunoreactive band at approximately 20 kDa, for which antibodies were generated in rabbit Ai but not Bii (Figure 4.6).

Antisera were also screened for antibodies against oIGFBP-4 by the ability of sera to immunoprecipitate ^{125}I-IGF cross-linked to oIGFBP-4. Both cross-linked ^{125}I-IGF-I and ^{125}I-IGF-II were tested, giving identical results with either complex. Results for ^{125}I-IGF-I/IGFBP-4 are shown. Using this screening procedure, antisera from rabbit Bi contained antibodies to oIGFBP-4, present by the second boost. The development of immunoreactivity against IGF cross-linked to oIGFBP-4 in rabbit Bi, throughout the immunisation protocol, is shown in Figure 4.7A. The titre appears to be maximal by the second boost and remains relatively constant throughout the immunisation protocol. A titration curve from the terminal bleed obtained from rabbit Bi, in comparison to that of an antibody to native oIGFBP-4, is shown in Figure 4.7B. By this screening method the peptide antibody, Bi, appears to have a low titre, although this is only approximately three fold lower than that of the polyclonal antibody raised against native purified oIGFBP-4. Since the cross-linking of IGF to IGFBP-4 has the potential to mask or alter possible antibody binding sites on IGFBP-4, the antisera produced were also tested for the ability to immunoprecipitate IGFBP-4 directly from plasma. Once again antisera from rabbit Bi was the only sera to contain significant amounts of immunoreactivity directed against plasma oIGFBP-4 (Figure 4.8A). Antisera Bi did not immunoprecipitate IGFBP-4 from bovine, pig, rat, human or chicken sera or plasma or from human (HE39L) or rat (L6) cell culture medium (Figure 4.8B). Since DNA construct A was directed against human IGFBP-4 with one amino acid change compared with oIGFBP-4 (Figure 4.1), antisera from Ai and Aii were tested for the ability to immunoprecipitate human IGFBP-4. Neither antisera contained activity capable of precipitating human IGFBP-4 from plasma (Figure 4.8B).

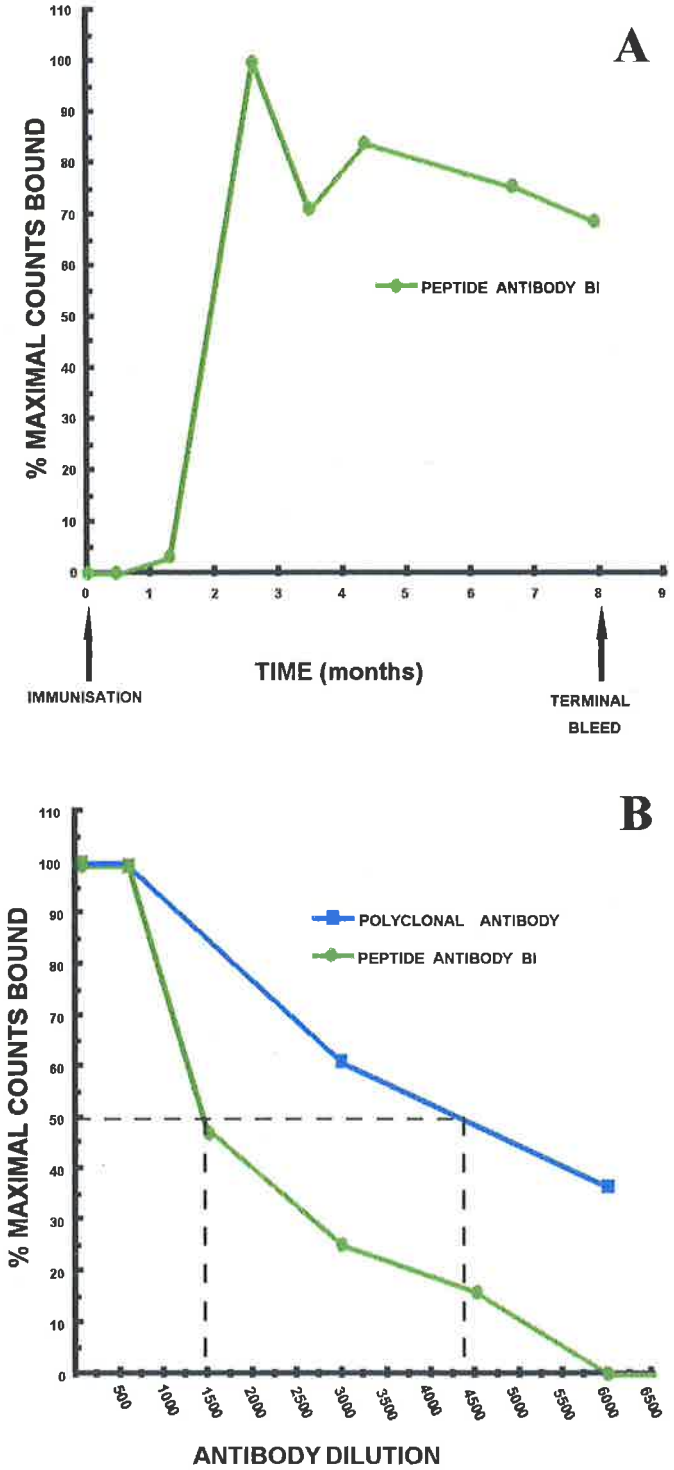


FIGURE 4.7 CHARACTERISATION OF IGFBP-4 ANTIBODY. ^{125}I-IGF-I was cross-linked to oIGFBP-4 and cross-linked monomers purified by gel filtration. ^{125}I-IGF-I / IGFBP-4 complexes were immunoprecipitated with antisera Bi, at a final dilution of 1/60 (A) or as indicated (B), (4.2.6.3). Radioactivity in the pellets was counted and specific binding expressed as a percentage of the maximal counts bound. A = time course of antibody production. B = titration curve compared to an oIGFBP-4 polyclonal antibody.

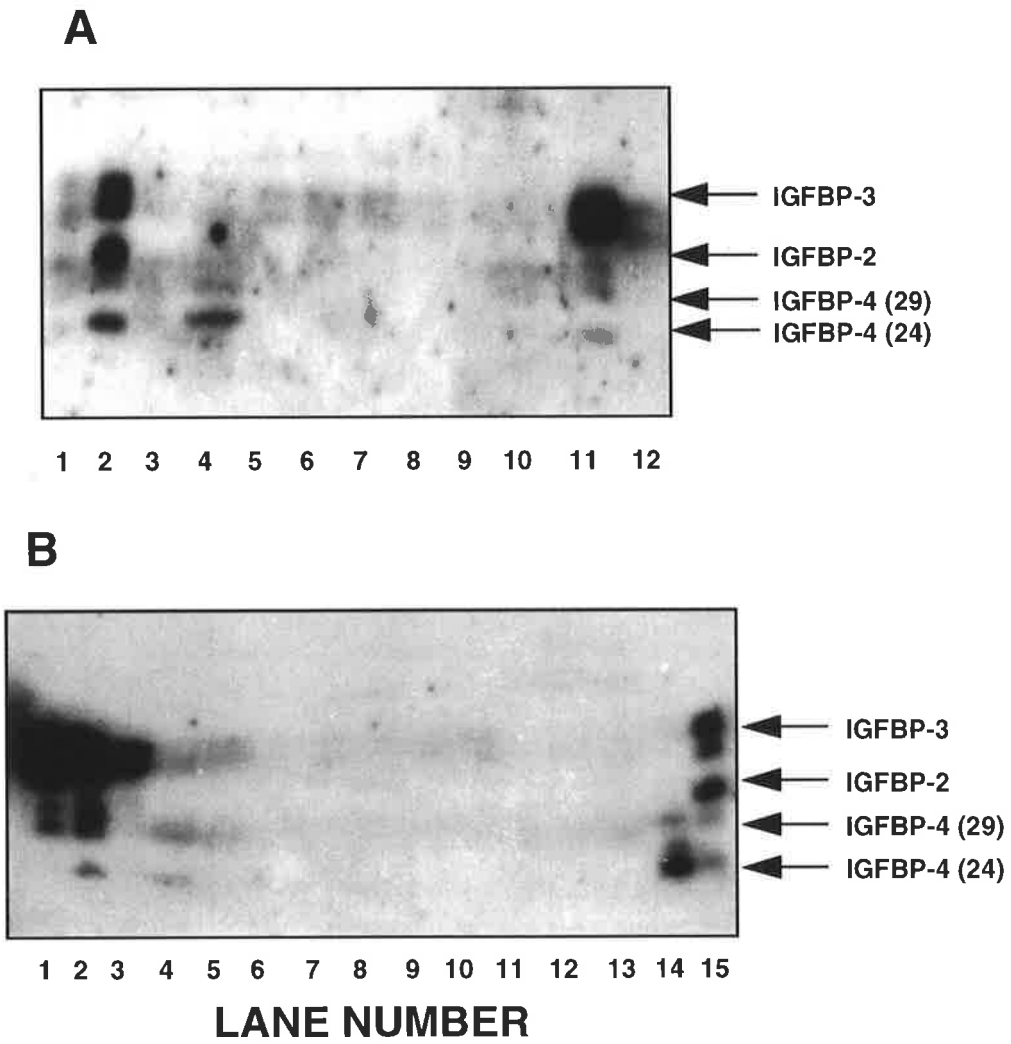


FIGURE 4.8 IMMUNOPRECIPITATION OF IGFBP-4 FROM PLASMA OR CULTURE MEDIA. Plasma or culture media was substituted for cross-linked IGFBP-4 and immunoprecipitated as described in 4.2.6.4. Precipitated proteins were subjected to non-reducing SDS-PAGE and Western ligand blotting (2.2.3.1). A = Immunoprecipitation of ewe plasma with antisera and pre-immune sera (PI). 1 = non-specific binding (NSB); 2 = un-precipitated ewe plasma (1 µl); 3 = PI Bi; 4 = Bi; 5 = PI Aii; 6 = Aii; 7 = PI Ai; 8 = Ai; 9 = PI Bii; 10 = Bii; 11 = un-precipitated human plasma (1 µl); 12 = NSB. B = Cross species immunoprecipitation. 1 = un-precipitated porcine plasma (1µl); 2 = un-precipitated ewe plasma (1 µl); 3 = un-precipitated human plasma (1µl); 4 = Bi + ewe plasma; 5 = Bi + bovine plasma; 6 = Bi + human plasma; 7 = Aii + human plasma; 8 = Ai + human plasma; 9 = Bi + rat plasma; 10 = Bi + porcine plasma; 11 = Bi + chicken plasma; 12 = Bi + L6 culture medium; 13 = Bi + HE39L culture medium; 14 = un-precipitated L6 culture medium (5 µl); 15 = un-precipitated bovine plasma (1µl). Possible IGFBP bands, including the two different molecular weight forms of IGFBP-4, are indicated.

4.4 DISCUSSION

This chapter describes the successful use of an alternative method to peptide conjugation for immunising with defined regions of a protein molecule. Peptide regions of interest in IGFBP-4 were chosen based on hydrophilicity profiles and predicted antigenicity. To generate a specific antibody, peptide regions of IGFBP-4 were also selected that had low homology to other IGFBPs, particularly IGFBP-2. Antigenicity profiles, as determined by the methods of Welling *et al.* (1985) and Jameson and Wolf, (1988), were not always in agreement. Therefore, the primary rationale for choosing peptide regions A and B of IGFBP-4, was based on IGFBP homologies, a hydrophilic nature and a high antigenicity by at least one method of prediction (Appendix I).

Peptide regions A and B introduced changes into the gene VIII product in terms of N-terminal charge and, for region A, also the potential for disulphide bonds. The introduction of these charged peptides occurs in between a hydrophobic portion of the leader peptide and the acidic mature N-terminus of the gene VIII protein. Both peptides A and B were successfully incorporated into hybrid bacteriophage particles. The production of hybrid bacteriophage particles suggest that cleavage of the leader peptide and packaging of the bacteriophage can still occur with these charged peptides inserted. However, the level of fusion products compared to wild type gene VIII particles was low, approximately 10 % for peptide B and 5 % for peptide A, as determined by laser densitometry of stained SDS-PAGE gels. This is lower than the 10 to 30 % incorporation of enlarged coat proteins reported by Greenwood *et al.* (1991). Thus, the generation of hybrid bacteriophage appears relatively flexible as to the nature of the peptide stretch introduced into the gene VIII product, although charged regions and disulphide bonds may reduce the level of hybrid protein incorporation. For peptide A, with cysteine residues, this may have been due to the formation of unfavourable inter-molecular disulphide bonds.

The method for generation of hybrid bacteriophage is relatively simple, although the lack of a selection method for single DNA inserts in the correct orientation requires large scale screening of possible recombinants by DNA sequencing. Prof. R. N. Perham and co-workers at the Department of Biochemistry, University of Cambridge, UK, have

recently improved the hybrid bacteriophage display system through the development of a modified vector pKfdH for directional insertion of DNA sequences into this system (Prof R. N. Perham, pers. comm.). The use of the hybrid bacteriophage display system generates a large amount of antigen, enough for immunisations of several animals with subsequent boosting. The DNA clone produced also represents a renewable source of antigen for future bacteriophage production and immunisations. In cases where there is a continual demand for large quantities of antibody (eg. large scale routine assays), this would provide a cost effective means, in comparison to repeated protein purifications or the purchase of conjugated peptides, for regenerating an antibody of defined specificity.

The hybrid bacteriophage preparation was highly antigenic, producing responses to fd proteins in all four rabbits by at least the second boost. Antisera were not tested for immunoreactivity against the introduced peptides. However, only one rabbit produced antibodies that recognised native oIGFBP-4. This variability of immune response is not uncommon when using rabbits to raise antibodies, due to their outbred nature (Muller, 1988).

The antibody produced against peptide B is specific for oIGFBP-4 and does not co-precipitate appreciable amounts of any other IGFBP from ovine plasma. Of particular note is the lack of cross-reactivity with oIGFBP-2. The antibody appears to recognise both the 24 kDa non-glycosylated and 29 kDa glycosylated forms of oIGFBP-4. The antibody is also species specific and does not precipitate IGFBP-4 from rat, porcine, bovine, human or chicken serum. This is not unexpected, since the corresponding sequences of peptide B for rat and human IGFBP-4 contain several amino acid substitutions, although bovine IGFBP-4 only differs at one position (Figure 4.1). The sera from the species listed above have lower levels of IGFBP-4 than sheep plasma (as detected by Western ligand blot) and thus this may also contribute to the apparent absence of immunoprecipitated IGFBP-4 in these species. However, culture media from a human (HE39L) and rat (L6) cell line, both of which produce large amounts of IGFBP-4, as judged by size and intensity by Western ligand blotting, also failed to show immunoprecipitation of IGFBP-4 with antisera against peptide B. Absolute confirmation of the species specificity of the antisera to peptide B

should be shown by immunoprecipitation of purified IGFBP-4 from species other than sheep, although, with the exception of human IGFBP-4 (Kiefer *et al.*, 1992), large amounts of IGFBP-4 protein are not readily available.

At the time that peptide regions A and B were selected, only partial sequence for oIGFBP-4 had been obtained which lacked the first 132 amino acids. The sequence for bovine IGFBP-4 was also not published. Consequently, DNA oligomers for region A were synthesised based on the sequence of human IGFBP-4, which was reasoned to be more homologous to oIGFBP-4 than that of the only other available IGFBP-4 sequence from rat. Unfortunately, as shown in Figure 4.1, there is a single amino acid substitution at position eight of peptide A in oIGFBP-4, resulting in an Arg (R) to Lys (K) change. Antibodies to oIGFBP-4 were not detected in antisera from rabbits immunised with peptide region A. The amino acid substitution at position eight, is conservative, maintaining positive charge, and therefore unlikely to be responsible for generating a species specific antibody against peptide A. A recent report describes generation of antibodies against this peptide region of rat IGFBP-4, which has an Arg (R) to His (H) substitution at position seven in comparison to peptide A, using traditional peptide conjugation (Liu *et al.*, 1993b). This indicates that site A is potentially antigenic. As described above, peptide A was not incorporated into the bacteriophage coat to as high a level as peptide B or other peptides displayed by Greenwood *et al.*, (1991). Therefore, the immunisation essentially presented less of peptide A than B to the immune system, which may have influenced the immune response. However, the most probable explanation for the lack of antibodies to peptide A is the variability of the immune response seen in rabbits (Muller, 1988). In future work, a larger number of rabbits should be immunised. Alternatively, antibodies to mammalian IGFBPs may be raised more efficiently in a species such as the chicken. The chicken has been reported to be different in many aspects of the IGF/IGFBP axes to mammals (Upton, 1993a). The predicted lower species homology between the antigen and endogenous IGFBPs may enhance the immune response. Additionally, the chicken is a useful model for generating antibodies due to the ability to harvest antibodies from the egg yolk (Polson *et al.*, 1980).

The titre of the antibody produced was modest, although comparisons with the polyclonal antibody prepared by Walton and Grant against native oIGFBP-4, suggests the affinity of the peptide antibody to be only approximately three fold lower. The apparent low titre may be due to the use of IGF cross-linked to IGFBP-4 as radioligand in the screening procedure, since the binding of IGF may in some way mask or alter epitopes of IGFBP-4. In addition, the radioimmunoprecipitation method was not optimal, since with either native or peptide IGFBP-4 antibody a maximum of only 10 to 15 % of added radioactivity was immunoprecipitable. Since the ^{125}I-IGF/IGFBP-4 complex was size fractionated to remove the majority of free IGF and larger cross-linked complexes, the large proportion of unprecipitable material is anomalous. However, structural alterations in the purified IGFBP-4 that do not affect IGF binding, ineffective precipitation of complexes by the secondary antibody or rapid degradation or aggregation of cross-linked tracer may explain the low precipitability of ^{125}I-IGF/IGFBP-4. The latter two explanations are less likely since the radioligand was used, on several occasions, immediately after production and antibody complexes appeared to be efficiently precipitated with native IGFBP-4 from plasma. Furthermore, experiments, not described in this chapter, failed to show immunoprecipitation of large amounts of purified oIGFBP-4 from the same batch of IGFBP-4 used for cross-linking, even though N-terminal sequence data, molecular size and IGF binding ability suggested the protein was native (data not shown). These technical problems must be rectified if this antibody is to be used to generate a reliable and specific RIA.

In conclusion, the use of a hybrid bacteriophage display system to present peptide regions of oIGFBP-4 as antigens has resulted in the production of an antibody to oIGFBP-4. The antibody produced is specific for oIGFBP-4 and has a titre of a similar order of magnitude to a polyclonal antibody generated against the native protein. This specific IGFBP-4 antibody may prove to be useful for the development of a specific assay to rapidly and reliably measure oIGFBP-4. The development of such an assay will aid further investigations of the roles and regulation of IGFBP-4. In addition, the hybrid bacteriophage display system may provide a useful means of generating a panel of specific

antibodies directed against known regions of IGFBPs which can subsequently be used in IGFBP structure/function studies, such as the identification of the IGF binding region of the IGFBP. The technique may also be applicable to the generation of antibodies for other IGFBPs from species where the large quantities of protein required for immunisations are not easily attainable.

CHAPTER 5. CIRCULATING IGFs, IGFBPs AND TISSUE mRNA FOR IGFBP-2 AND -4 IN THE OVINE FETUS

5.1 INTRODUCTION

The insulin-like growth factor binding proteins (IGFBPs) are a family of at least 6 highly conserved and structurally related proteins that specifically bind the IGFs (Rechler, 1993; Baxter, 1993). The roles of the IGFBPs are not clearly defined, although they have been shown to regulate the half-life of circulating IGFs, to modulate IGF activity (either an enhancement or inhibition) and are suggested to affect the distribution and tissue localisation of IGF (Rechler, 1993). Both the IGFs and IGFBPs have been suggested to be important factors for normal fetal growth and development, primarily due to recent gene knock-out experiments for the IGFs and the developmental changes observed in these proteins (1.3.2 and 1.3.3).

In the rat, primate or pig, circulating IGFBP-2 is higher in the fetus than in the adult (Donovan *et al.*, 1989; Liu *et al.*, 1991; Lee *et al.*, 1993a). In the fetal sheep or human the majority of circulating IGF is carried by the smaller molecular weight IGFBPs or the circulating type II IGF receptor (Butler and Gluckman, 1986; Wang and Chard, 1992), while in the adult mammal, IGFBP-3, in the form of the circulating 150 kDa, high molecular weight complex, is the major carrier of circulating IGF (D'Ercole *et al.*, 1980; Butler and Gluckman, 1986; Wang and Chard, 1992). While this suggests a specific role for IGFBP-2 in the fetus, recent gene knock-out experiments of IGFBP-2 in the mouse have resulted in no major phenotypic effect on fetal growth and development (Pintar *et al.*, 1994). The ontogeny of IGFBP-2 and IGF-II mRNA has recently been described in the ovine fetus, with co-ordinate changes in the abundance of IGF-II and IGFBP-2 mRNAs throughout development (Delhanty and Han, 1993). However, the ontogeny of IGFBP-4, which is suggested by observations in Chapter 3 to be developmentally regulated at the mRNA level, has not previously been investigated in any species. Additionally, much of the data describing the ontogeny of circulating IGFBPs is derived from studies in the late gestation

rat, in which fetal development is clearly different to that of larger mammalian species. Thus, the studies in this chapter have more fully characterised the developmental changes in IGFBPs throughout gestation, not only for IGFBP-2, but also for IGFBP-3 and IGFBP-4, in a larger mammalian species. In particular, the ontogeny of circulating IGFBPs as well as IGFBP mRNA levels in tissues have been examined and related to circulating IGFs.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

1,1,2-trichlorotrifluoroethane (Freon) was obtained from Ajax Chemicals, Sydney, NSW, Australia. Trimethylamine was obtained from FSE, Homebush, NSW, Australia. Purified ovine IGFBP-3 was generously donated by Mrs P. A. Grant, CRC for Tissue Growth and Repair, Adelaide, South Australia. Bovine IGFBP-2 was purified in our laboratory by Ms. J. A. Moss. Rabbit anti-bovine IGFBP-2 polyclonal antibody was generated by Mr. L. Szabo in our laboratory. Rabbit anti-ovine IGFBP-3 polyclonal antibody was generated by Mrs P. A. Grant and Dr. P. E. Walton, CRC for Tissue Growth and Repair, Adelaide, South Australia. Rabbit anti-Long R^3-IGF-I polyclonal antibody was provided by GroPep Ltd, Adelaide, South Australia. Long R^3-IGF-I is an analogue of IGF-I with glutamate (E) at position three replaced by arginine (R) and an N-terminal extension comprising the first 11 amino acids of porcine growth hormone. Antibodies against this analogue strongly react with IGF-I. Mouse anti-rat IGF-II monoclonal antibody was obtained from Amano Pharmaceutical Co. Ltd, Nagoya, Japan. Sheep anti-rabbit IgG, sheep anti-mouse IgG and rabbit IgG were all obtained from Silenus Laboratories, Hawthorn, Vic., Australia. Recombinant human IGF-II was provided by GroPep Pty Ltd, Adelaide, South Australia and recombinant human IGF-I was provided by Genentech, San Francisco, CA, USA. Lethabarb (sodium pentobarbitone, 325 mg/ml) was from Arnolds of Reading, Pty. Ltd., Peakhurst, NSW, Australia.

5.2.2 BLOOD AND TISSUE SAMPLING

Blood was collected via permanent in-dwelling umbilical vein catheters from fetuses 80 to 130 days and also from three fetuses at 140 days gestation. Catheters were inserted and blood samples collected by co-workers at the Department of Obstetrics and Gynaecology and Department of Physiology, University of Adelaide, South Australia, as described by Robinson *et al.* (1979). Cord blood from un-catheterised fetuses at 45, 60, 140 and 145 day gestation was collected at post-mortem with the assistance of Mr. F. Carbone, Ms. T. De Barrow or Ms. L. Mundy, Department of Obstetrics and Gynaecology, University of Adelaide, South Australia. Blood from lambs less than 24 hours old was collected by jugular venipuncture by Ms. T. Heard, South Australian Department of Agriculture, Turretfield Research Station, Turretfield, South Australia. In all cases the blood was collected into heparinised tubes and plasma prepared by centrifugation at 4,000 x g for 20 minutes. Plasma was aliquotted and stored at -20° C until analysis. Samples were as follows: 45 days (n = 2); 60 days (n = 4); 80 days (n = 5); 95 days (n = 5); 120 days (n = 10); 130 days (n = 8); 140 days (n = 5); 145 days gestation (n = 3); 1 day post-natally (n = 6) and normal adult ewe plasma (NEP) (n = 3, pooled). Sheep plasma used to assess the effect of surgery on circulating fetal IGFBPs (Appendix II) was obtained from a different pool of adult sheep to the normal ewe plasma (NEP) that was used in subsequent experiments. Animals were sacrificed by pentobarbitone euthanasia injection (Lethabarb, 0.5 ml/kg body weight) into the carotid artery of the pregnant ewe or 1 day lamb. Tissues were removed immediately, snap frozen into liquid nitrogen and stored at -80° C until analysis. Samples were as follows: 30 days (n = 6, pooled, livers and hearts only); 45 days (n = 1); 60 days (n = 2); 95 days (n = 2); 120 days (n = 2); 130 days (n = 3); 145 days gestation (n = 3) and 1 day post-natally (n = 3). Human and rat plasma were as described in section 4.2.1.4.

5.2.3 WESTERN LIGAND BLOTTING

Samples (20 µl of a 1 in 10 dilution in PBS, pH 7.5) were subjected to non-reducing, discontinuous SDS-PAGE and Western ligand blotting, using ^{125}I-IGF-II, as

described in 2.2.3.1. Filters were exposed to x-ray film at -80° C with intensifying screens for two to three weeks. A sample from a pool of normal ewe plasma (NEP) was co-electrophoresed in triplicate in each experiment as a control. Each experiment was duplicated. Autoradiographs were subjected to densitometric scanning (Molecular Dynamics Computing Densitometer, Model 300A, Imagequant^{TM} version 3.22) and each band expressed as a percentage of the average of the 42 kDa band (IGFBP-3, lower band) of the NEP control. The inter- and intra-assay co-efficients of variation (CV) were 13.45 %, and 16.1 % ± 1.65 for IGFBP-2 and 22 % and 16.72 % ± 2 for IGFBP-4, respectively. The intra-assay CV for IGFBP-3 was 13.76 % ± 1.25. An inter-assay CV for IGFBP-3 was not calculated since direct comparisons between experiments is not valid due to variations in experimental conditions, such as the ^{125}I-IGF-II batch or age and autoradiograph exposure times. To allow relative comparisons between experiments, the lower band of IGFBP-3 was arbitrarily assigned as 100 % binding.

Fractions from acid-gel chromatography of fetal plasma were concentrated, neutralised, with five volumes of 1 M Tris pH 7.5, and subjected to Western ligand blotting, as above. Molecular weights of IGFBP bands were calculated from a standard curve of migration distances of ^{14}C molecular weight markers (data not shown).

5.2.4 OVINE IGFBP-3 RADIOIMMUNOASSAY

Plasma (20 µl) was acidified with an equal volume of 0.8 M acetic acid, pH 2.8, 1.2 % (v/v) trimethylamine, 2 % (v/v) Tween-20, and incubated at room temperature for 20 minutes. Samples were then neutralised with 0.6 volumes of 0.4 M Tris, diluted to 1 ml and 50 µl subjected to IGFBP-3 radioimmunoassay (RIA), developed as previously described for porcine IGFBP-3 (Walton and Etherton, 1989). Purified ovine IGFBP-3 (Carr *et al.*, 1994a) was used to generate a rabbit polyclonal antibody with a titre of 2,400. The RIA was performed as described by Walton and Etherton (1989) with the exception that radiolabelled IGF-I-IGFBP-3 was prepared as described by Baxter and Martin (1986). This assay exhibited parallel displacement with ovine fetal, maternal and adult plasma and purified ovine IGFBP-3, with an ED_{50} of approximately 1 µg per assay tube (300 µl).

Furthermore, the assay was specific for oIGFBP-3, exhibiting no cross reactivity with IGF-I, IGF-II, oIGFBP-4, human IGFBP-3, porcine IGFBP-3, or human, rat, porcine and bovine plasma. The RIA had an inter-assay CV of 16.8 % for 12 assays and intra-assay CV of <15 %. The oIGFBP-3 RIA was developed and assays performed by Mrs. P. A. Grant and Dr. P. E. Walton, CRC for Tissue Growth and Repair, Adelaide, South Australia.

5.2.5 NORTHERN ANALYSIS

Total RNA was extracted from adult or fetal sheep tissues using the method of Chomczynski and Sacchi (1987), with slight modification, as described in 2.2.2.1. RNA (50 µg) was subjected to Northern analysis for IGFBP-2 and IGFBP-4 mRNA and 18S rRNA, as described in 2.2.2.2. Filters were exposed to x-ray film with intensifying screens at -80^{o} C for three days (IGFBP-2 or IGFBP-4 in the liver or kidney), 14 days (IGFBP-2 heart or lung or IGFBP-4 in the heart), seven days (IGFBP-4 in the lung) or overnight (18S rRNA). RNA transcripts were sized by co-electrophoresis of an RNA ladder. The RNA ladder was then visualised by probing the membrane with λ DNA and transcript size determined by construction of a standard curve of distance migrated from origin (data not shown).

5.2.6 IGF-I AND IGF-II RADIOIMMUNOASSAY

IGFs were dissociated from IGFBPs prior to RIA by acid-gel chromatography. Plasma samples (120 µl) were diluted to 20 % (v/v) plasma with mobile phase (0.2 M acetic acid [v/v] pH 2.5, 0.3% [v/v] trimethylamine, 0.5% [v/v] Tween-20) and extracted with an equal volume of Freon. The aqueous phase was filtered (0.2 µm cellulose acetate filters, Alltech Associates Australia Pty Ltd., Homebush, NSW, Australia) by centrifugation at 2500 x g for two minutes and 200 µl of the filtrate was injected onto a Protein-Pak 125 size exclusion column (Waters/Millipore, Lane Cove, NSW, Australia) at a flow rate of 1 ml/min using a AS200 Automatic Injector (GBC Scientific Equipment, Marleston, South Australia). To establish immunoreactivity elution profiles, 0.25 minute fractions were collected from 5.5 to 11.5 minutes of elution and 50 µl of each fraction subjected to RIA after neutralisation with 0.6 volume of 0.4 M Tris. For analysis of total IGF-I, fractions were

collected from 7.75 to 10 minutes of elution and 50 µl of each fraction neutralised, as above, and analysed by RIA. For analysis of IGF-II, fractions were diluted 1/4, neutralised and assayed as for IGF-I. Recombinant human IGF-I and IGF-II were used as standards and radioligand. The IGF-I RIA utilised a rabbit anti-human IGF-I polyclonal antibody at a final dilution of 1/80,000. The IGF-II RIA used a mouse anti-rat IGF-II monoclonal antibody at 1 ng per reaction tube. Cross reactivities of recombinant human IGF-II with rabbit anti-human IGF-I antibody was less than 1 % and recombinant human IGF-I with mouse anti-rat IGF-II monoclonal antibody was 2.5 %. Ovine IGF-I and IGF-II are only one and four amino acids different from their respective human peptides and equipotent with either human or bovine growth factors in bioassays, receptor and IGFBP binding assays and radioimmunoassays (Francis *et al.*, 1989). Sensitivity of the IGF-I assay was 2.6 ng /ml plasma. Intra- and inter-assay CVs were 7.7 ± 1.6 % and 11.3 % respectively. Sensitivity of the IGF-II assay was 3.4 ng /ml plasma. Intra- and inter-assay CVs were 5.5 ± 1 % and 5.7 % respectively. Total IGF-I or IGF-II was calculated by the summation of fractions from 8.5 to 10 minutes elution time. The IGF-I RIA used the same standards and radioligand, but different antibody to human IGF-I, to that previously validated for ovine IGF-I (Francis *et al.*, 1989). Similar results were obtained using both these, and a third, rabbit anti-human IGF-I serum (Owens *et al.*, 1994). The validity of the IGF-II RIA was demonstrated using bovine IGF-II, identical to ovine IGF-II with the exception of a Thr (T) to Ala (A) substitution at amino acid position 62. Bovine IGF-II showed parallel displacement to rat and human IGF-II. To further validate the ovine IGF-II RIA, plasma from 12 ovine fetuses was subjected to acid-gel chromatography. The activity of the IGF containing column fractions was assayed using the IGF-II RIA and a homologous IGF-II radioreceptor assay which had been previously calibrated with ovine IGF-II (Francis *et al.*, 1989). There was a close correlation between by the two assay methods (r^2=0.93). This assay validation was performed by Mrs. K. J. Quinn, CRC for Tissue Growth and Repair, Adelaide, South Australia. Acid-gel chromatography was performed with the aid of Mrs. K. J. Quinn and Ms. K. Irvine, CRC for Tissue Growth and Repair, Adelaide, South Australia.

5.2.7 ^{125}I-IGF-I BINDING OF ACID-GEL FILTRATION FRACTIONS

Fetal plasma from 60 and 145 days gestation was subjected to acid-gel filtration, as described in 5.2.6. Fractions from 6.5 to 7.75, 8.25 to 8.5 and 8.75 to 10 minutes elution time, representing peak I, the shoulder region and peak II, respectively (see Figure 5.11), were pooled. Three nanograms of immunoreactivity from each pool was concentrated and neutralised with five volumes of 1 M Tris, pH 7.5 then incubated with approximately 10,000 cpm of ^{125}I-IGF-II, overnight at 4^{o} C. Reactions were subjected to neutral gel filtration, as described in section 7.2.4, at a flow rate of and 0.3 ml/min and one minute fractions collected. The radioactivity in each fraction was assessed by counting (LKB Wallac 1261 Multigamma Counter, LKB, Turku, Finland).

5.2.8 STATISTICAL ANALYSIS

The relationships between different variables and gestational age were analysed by regression analysis, using linear or quadratic models (Proc GLM, SAS, SAS Institute Inc. Cary, NC, USA). The relationship between liver IGFBP-2 mRNA and gestational age was analysed by regression analysis with a segmented model (Cricket Software, Great Valley Corporate Centre, Malvern, PA, USA). Relationships between variables were examined by correlation analysis (Proc Corr, SAS, SAS Institute Inc. Cary, NC, USA), in which data *in utero* was considered alone, as well as with data obtained from 1 day post-natal animals.

5.3 RESULTS

5.3.1 THE EFFECT OF SURGERY ON CIRCULATING IGFBPs

The validity of using plasma samples from catheterised fetuses was established by demonstrating stability of IGFBP levels following surgery. Data from Western ligand blot analysis of circulating IGFBPs in samples taken from catheterised fetuses from one to 10 days post-surgery is presented in Appendix II. Some fetuses showed slightly higher IGFBP levels at one day post-surgery, while plasma from one fetus had markedly high levels of an IGFBP of approximately 30 kDa, perhaps IGFBP-1, at one day post-surgery. However, levels of IGFBPs were relatively static by two to five days post-surgery, suggesting that the

catheterised fetuses had recovered from any surgery-induced effect on circulating IGFBPs (Appendix II). All subsequent analysis used plasma samples taken from fetuses at least five days after surgery.

5.3.2 CIRCULATING IGFBPs IN THE OVINE FETUS

Circulating IGFBPs from 45 to 145 days gestation and 1 day post-natally were analysed by Western ligand blotting. A representative gel showing plasma profiles across these ages is shown in Figure 5.1. The binding species evident are a doublet at approximately 42 to 50 kDa, 33 kDa and 24 kDa. Additionally, other minor bands below 33 kDa and at approximately 200 kDa can be visualised. Using Western immunoblotting the 33 kDa band was shown to be ovine IGFBP-2 (Figure 5.2). The 42 to 50 kDa doublet and 24 kDa band were termed IGFBP-3 and IGFBP-4, respectively, in accordance with previous reports (Walton *et al.*, 1990; Gallaher *et al.*, 1992; Fowlkes and Freemark, 1992b). Plasma samples were subjected to Western ligand blotting and compared to a normal adult ewe plasma (NEP) pool run in triplicate on each gel. Each gel was run in duplicate. The intensity of bands obtained by densitometric scanning of autoradiographs is presented in Figure 5.3 and, in the absence of major post-translational modifications, substantially reflects plasma IGFBP protein levels. Circulating IGFBP-2 increased with age to peak at 120 days, then declined by 1 day post-natally ($p=0.002$). Circulating IGFBP-4 increased linearly between 45 and 145 days gestation ($p=0.004$). Circulating IGFBP-3, as determined by Western ligand blotting, increased with age ($p=0.04$). Figure 5.4 shows circulating IGFBP-3 throughout gestation, as measured by RIA. This more precise assay for IGFBP-3 also shows an increase in circulating IGFBP-3 with advancing gestational age ($p=0.0001$). Circulating IGFBP-3 measured by Western ligand blotting was positively correlated with immunoreactive IGFBP-3 quantitated by RIA ($r=0.55$, $p=0.0006$).

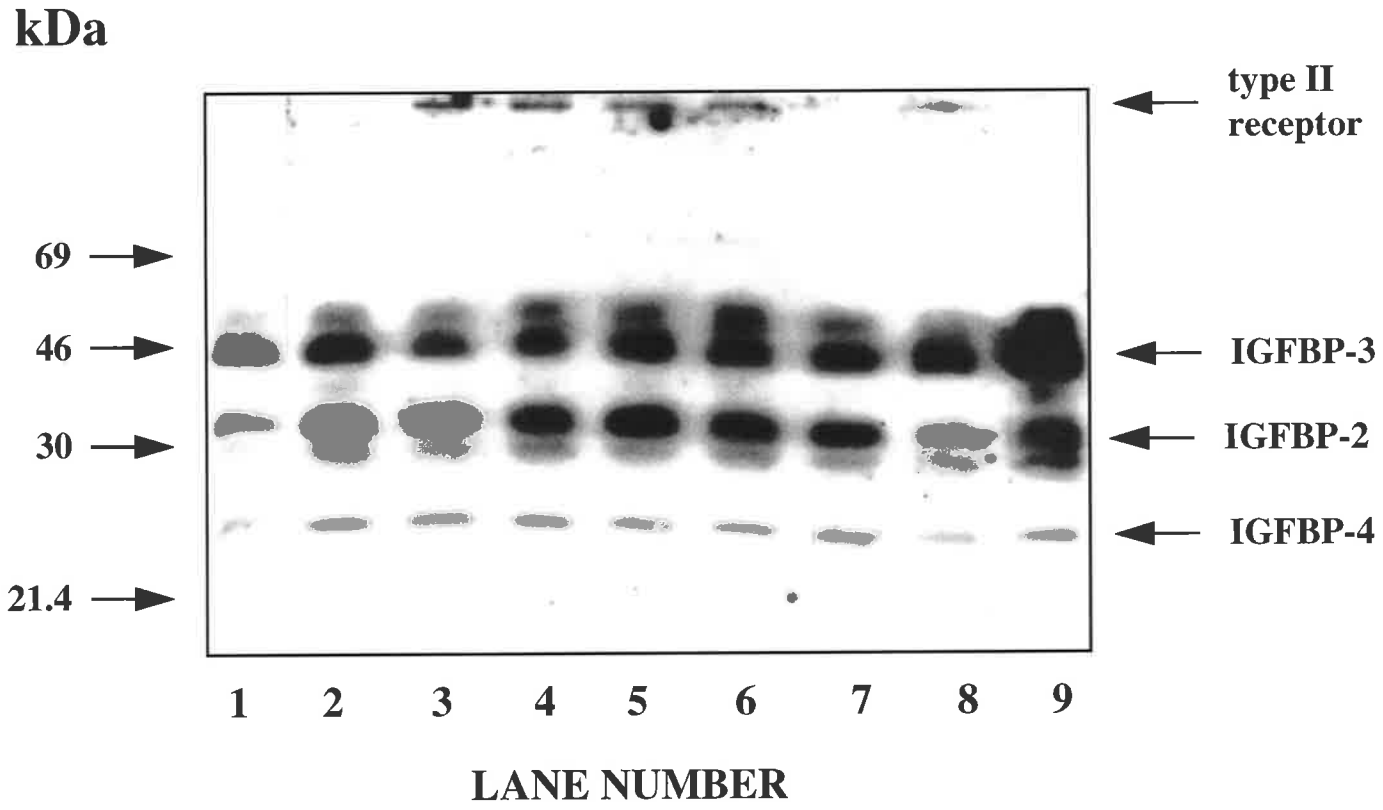


FIGURE 5.1 WESTERN LIGAND BLOTTING OF FETAL SHEEP PLASMA. Plasma (2 µl equivalents), from fetal sheep throughout gestation, were subjected to Western ligand blotting as described in 5.2.3. Lane 1 = 45 days; Lane 2 = 80 days; Lane 3 = 95 days; Lane 4 = 120 days; Lane 5 = 130 days; Lane 6 = 140 days; Lane 7 = 145 days gestation; Lane 8 = 1 day post-natal lamb; Lane 9 = normal adult ewe plasma (NEP). Estimated molecular weights and identifications of the IGFBP bands are indicated.

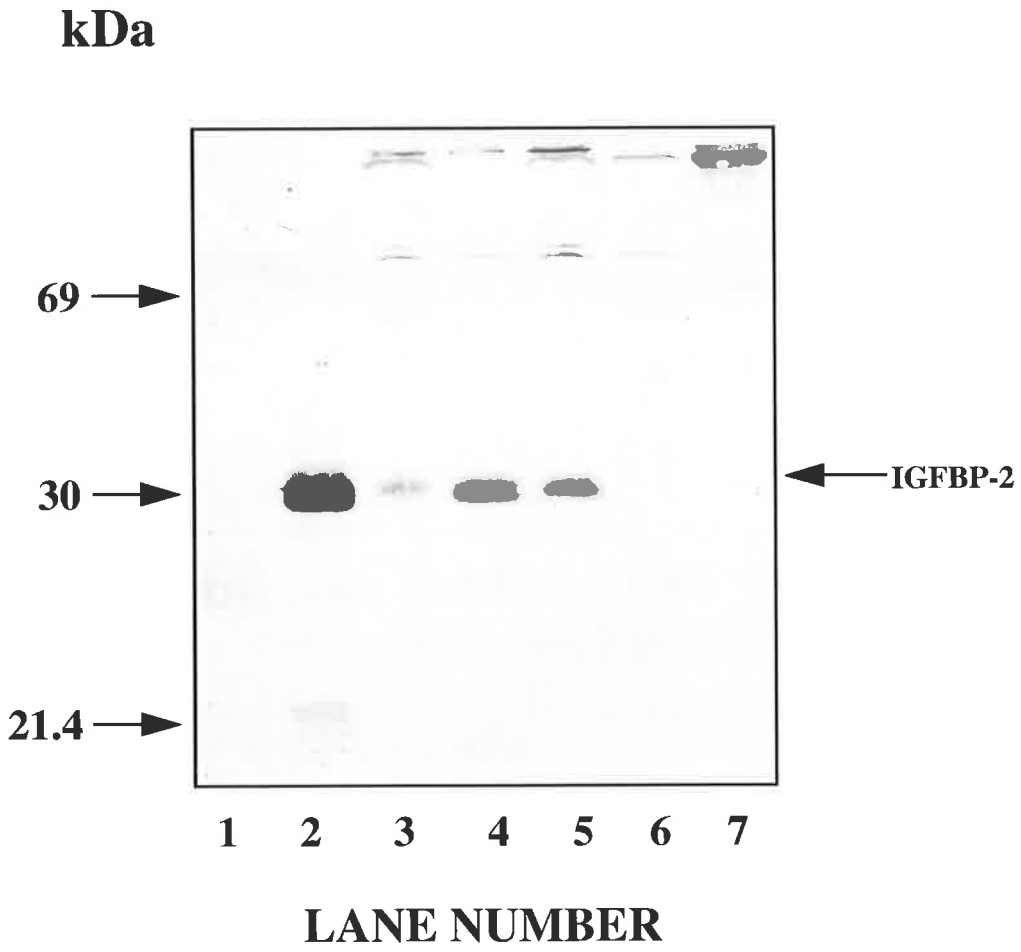


FIGURE 5.2 WESTERN IMMUNOBLOTTING OF PLASMA FOR IGFBP-2. Plasma was subjected to non-reducing SDS-PAGE, Western transfer and immunoblotting using a bovine IGFBP-2 antibody at 1 / 500 dilution, as described in 2.2.3.2. Lane 1 = 25 ng ovine IGFBP-3; Lane 2 = 30 ng bovine IGFBP-2; Lane 3 = normal adult ewe plasma (NEP); Lane 4 = 120 days gestation ovine fetal plasma; Lane 5 = 120 days gestation pregnant ewe plasma; Lane 6 = adult rat serum; Lane 7 = adult human plasma. Molecular weight markers and the position of immunoreactive IGFBP-2 is indicated.

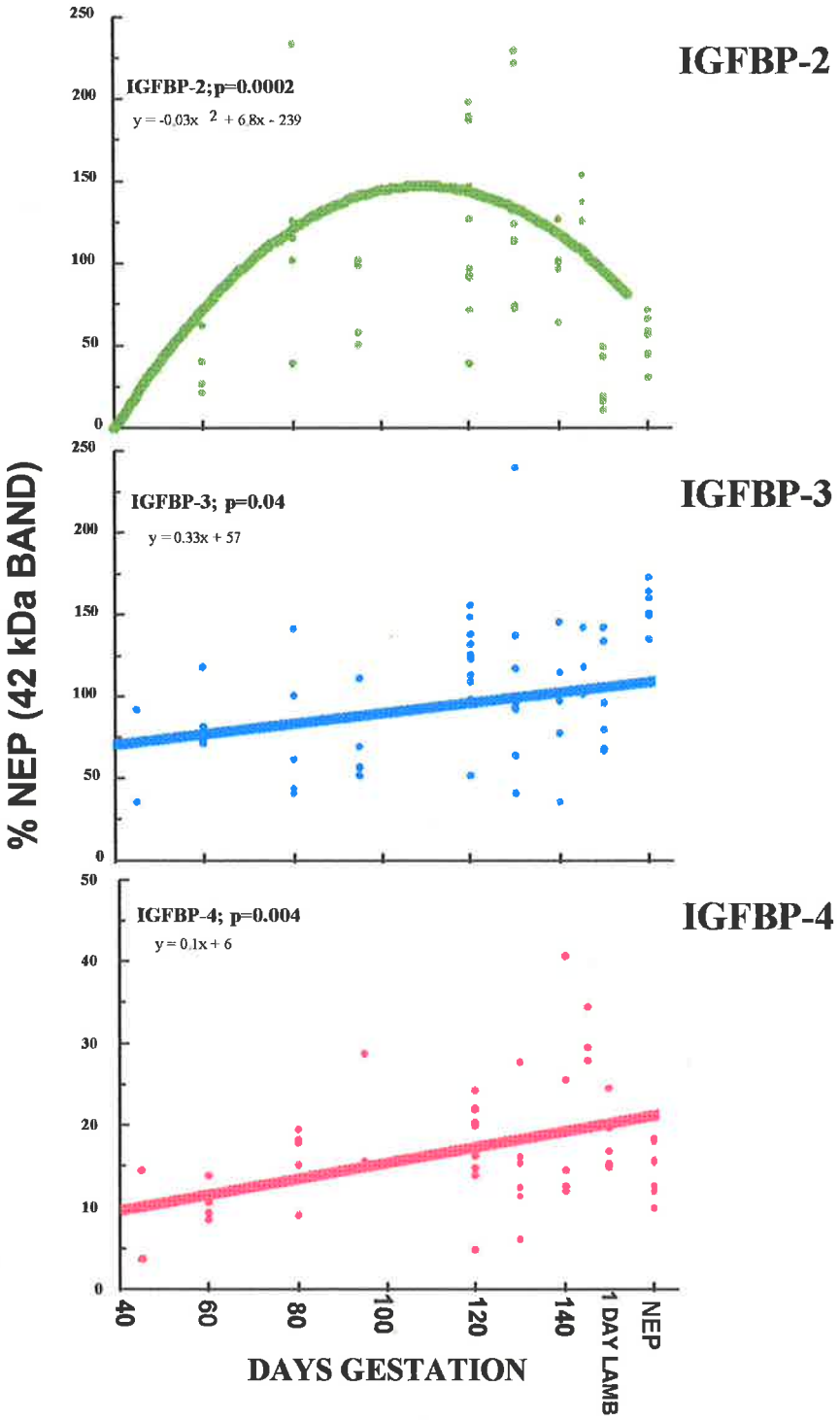


FIGURE 5.3 QUANTITATION OF CIRCULATING IGFBPs BY WESTERN LIGAND BLOT. Plasma samples were subjected to Western ligand blotting as described in section 5.2.3. A control (pooled normal adult ewe plasma; NEP) was electrophoresed in triplicate on each gel and gels were duplicated. Autoradiographs were scanned and band intensities expressed as a percentage of NEP IGFBP-3 42 kDa band. 45 days, n = 2; 60 days, n = 4; 80 days, n = 5; 95 days, n = 5; 120 days, n = 10; 130 days, n = 8; 140 days, n = 5; 145 days gestation, n = 3; 1 day post-natally, n = 6. Data was subjected to regression analysis using linear or quadratic models. P (probability) values and regression lines are shown.

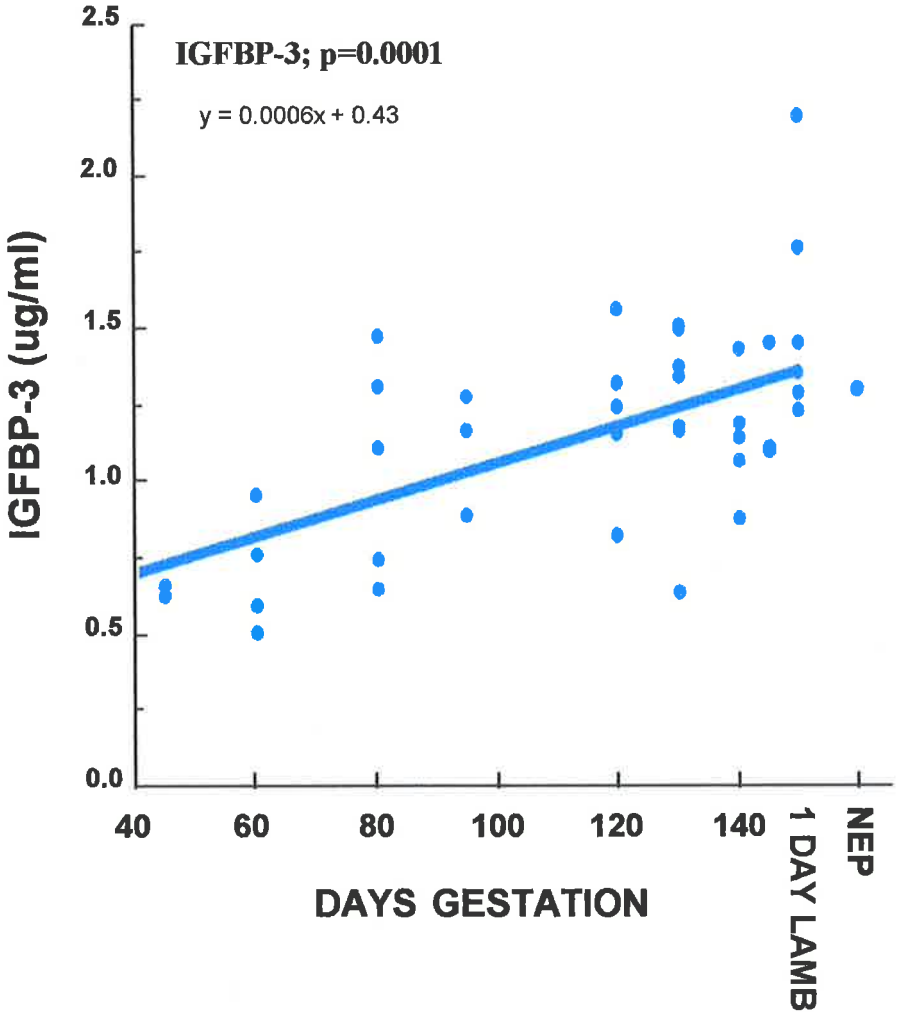


FIGURE 5.4 QUANTITATION OF CIRCULATING IGFBP-3 BY RIA. Plasma samples were analysed by ovine IGFBP-3 RIA as described in 5.2.4. Values for n are as described in Figure 5.3, except 95 days, n = 4; 120 days, n = 6; 130 days, n = 7. NEP = normal adult ewe plasma. Data was analysed by linear regression. P values and regression lines are shown.

5.3.3 TISSUE mRNA LEVELS ENCODING IGFBP-2 AND IGFBP-4

Northern analysis was used to investigate if the circulating changes in IGFBPs were reflected by changes in steady state IGFBP mRNA levels in the major fetal organs (heart, liver, lung or kidney). Northern analysis of IGFBP-3 mRNA in fetal liver is shown in Figure 5.5. As can be seen in Figure 5.5 the quality of the hybridising signal is poor. Additionally, detection of ovine IGFBP-3 mRNA was not reliable and thus, unfortunately, IGFBP-3 could not be investigated with this method using the rat IGFBP-3 cDNA probe available (Albiston and Herington, 1990). Tissues were also analysed for IGFBP-2 and IGFBP-4 mRNA. For IGFBP-2 a single band of approximately 1.6 kb was observed. For IGFBP-4, a major band of approximately 2.6 kb and two minor bands of 2.1 and 1.8 kb were observed, as previously reported (Carr *et al.*, 1994a, Chapter 3). Representative gels showing ontogenic changes in IGFBP-2 and IGFBP-4 in the liver, kidney, lung and heart are shown in Figure 5.6 to 5.9, respectively. Autoradiographs were analysed by densitometric scanning, expressed as a percentage of 18S rRNA and finally normalised with respect to a normal ewe liver sample co-electrophoresed in each experiment (Figure 5.10). In the liver, IGFBP-2 and IGFBP-4 mRNAs increased between 30 and 145 days gestation ($p<0.001$ and $p=0.04$, respectively). IGFBP-2 liver mRNA then declined after 145 days gestation ($p<0.01$, Figure 5.10). In the kidney, neither IGFBP-2 nor IGFBP-4 mRNA varied with age (Figure 5.10), although IGFBP-2 mRNA in the kidney appears to parallel changes in liver IGFBP-2 mRNA. In the heart, both IGFBP-2 and IGFBP-4 mRNAs declined with advancing gestation ($p=0.0001$ and $p=0.001$, respectively), (Figure 5.10). In late gestation, mRNA for IGFBP-2 or IGFBP-4 is barely detectable in the heart. IGFBP-2 mRNA in the lung decreased with advancing fetal age, in a similar manner to that seen for IGFBP-2 mRNA in the heart ($p=0.0001$). IGFBP-4 mRNA in the lung did not vary with age (Figure 5.10). In mid to late gestation, when circulating IGFBP-2 is most abundant, IGFBP-2 mRNA is highest in the kidney > liver >> heart = lung. At late gestational ages, when circulating IGFBP-4 is maximal, IGFBP-4 mRNA is highest in liver ≥ kidney > lung >> heart.

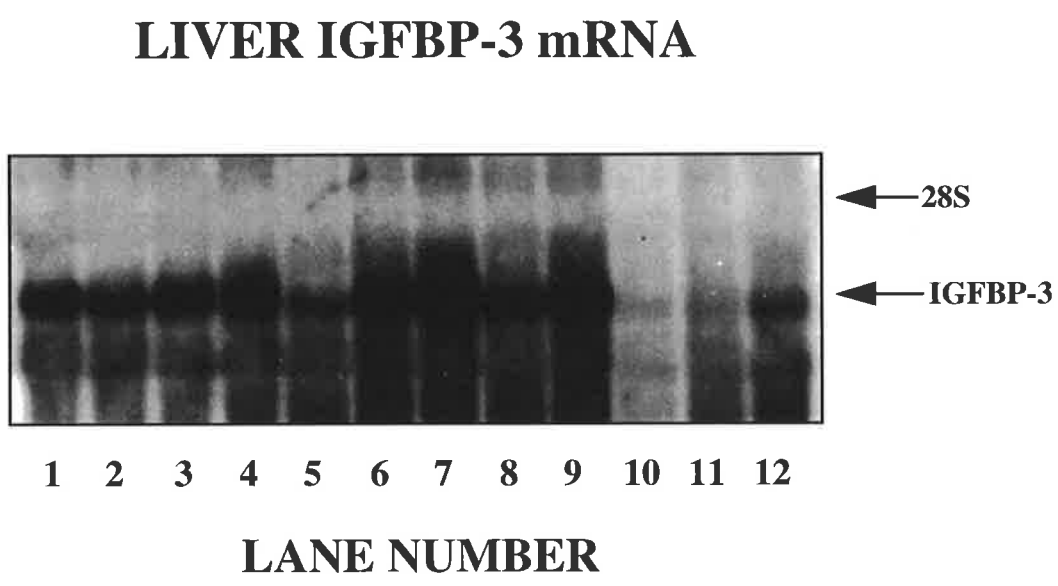
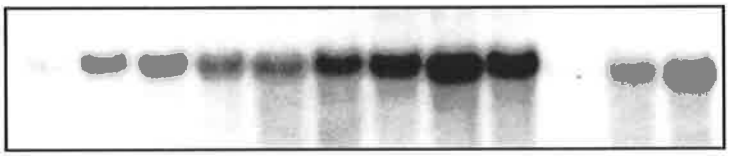


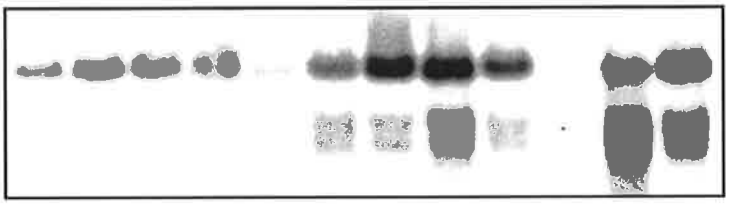
FIGURE 5.5 NORTHERN ANALYSIS OF IGFBP-3 mRNA IN FETAL LIVER. Fetal liver RNA was extracted and subjected to Northern analysis, as described in section 5.2.5. The filter was probed for IGFBP-2 and IGFBP-4 (see Figure 5.6), stripped in boiling water and reprobed with a rat IGFBP-3 DNA insert (Albiston and Herington, 1990) at 37° C in 40 % (v/v) deionised formamide. Filters were washed up to 55° C in 2 X SSC, 0.1 % (w/v) SDS and autoradiographed at -80° C for 4 weeks, with intensifying screens. Lane 1 = 30 days gestation; Lanes 2 and 3 = 60 days; Lane 4 = 95 days; Lane 5 = 120 days; Lanes 6 and 7 = 130 days; Lanes 8 and 9 = 145 days; Lanes 10 and 11 = 1 day post-natal lamb; Lane 12 = adult ewe liver. The positions of the IGFBP-3 transcript of undetermined size, and 28S rRNA are indicated.

LIVER

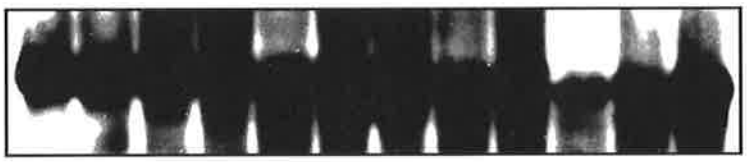
IGFBP-2



IGFBP-4



18S rRNA



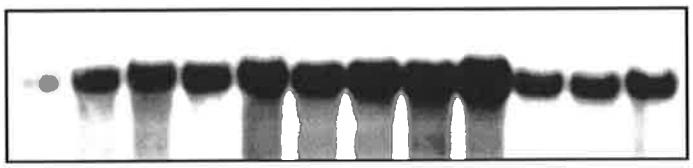
1 2 3 4 5 6 7 8 9 10 11 12

LANE NUMBER

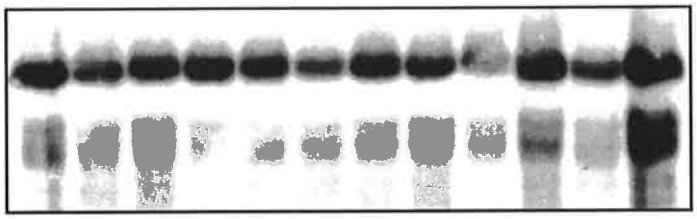
FIGURE 5.6 IGFBP-2 AND IGFBP-4 mRNA IN THE FETAL LIVER. RNA was extracted and subjected to Northern analysis, as described in 5.2.5. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA, stripping the bound probe in boiling water in between hybridisations. Representative gels from a subset of animals are shown. Lane 1 = 30 days gestation; Lanes 2 and 3 = 60 days; Lane 4 = 95 days; Lane 5 = 120 days; Lanes 6 and 7 = 130 days; Lanes 8 and 9 = 145 days; Lanes 10 and 11 = 1 day post-natal lamb; Lane 12 = normal adult ewe liver (control).

KIDNEY

IGFBP-2



IGFBP-4



18S rRNA

1 2 3 4 5 6 7 8 9 10 11 12

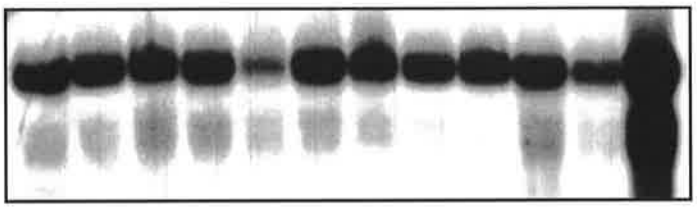
LANE NUMBER

FIGURE 5.7 IGFBP-2 AND IGFBP-4 mRNA IN THE FETAL KIDNEY. RNA was extracted and subjected to Northern analysis, as described in 5.2.5. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA, stripping the bound probe in boiling water in between hybridisations. Representative gels from a subset of animals are shown. Lane 1 = normal adult ewe kidney; Lanes 2 and 3 = 60 days gestation; Lane 4 = 95 days; Lane 5 = 120 days; Lanes 6 and 7 = 130 days; Lanes 8 and 9 = 145 days; Lane 10 and 11 = 1 day post-natal lamb; Lane 12 = normal adult ewe liver (control).

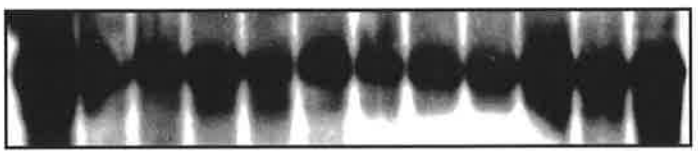
LUNG

IGFBP-2

IGFBP-4



18S rRNA



1 2 3 4 5 6 7 8 9 10 11 12

LANE NUMBER

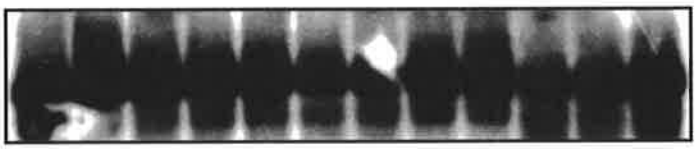
FIGURE 5.8 IGFBP-2 AND IGFBP-4 mRNA IN THE FETAL LUNG. RNA was extracted and subjected to Northern analysis, as described in 5.2.5. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA, stripping the bound probe in boiling water in between hybridisations. Representative gels from a subset of animals are shown. Lane 1 = normal adult ewe lung; Lanes 2 and 3 = 60 days gestation; Lane 4 = 95 days; Lane 5 = 120 days; Lanes 6 and 7 = 130 days; Lanes 8 and 9 = 145 days; Lanes 10 and 11 = 1 day post-natal lamb; Lane 12 = normal adult ewe liver (control).

HEART

IGFBP-2

IGFBP-4

18S rRNA



1 2 3 4 5 6 7 8 9 10 11 12

LANE NUMBER

FIGURE 5.9 IGFBP-2 AND IGFBP-4 mRNA IN THE FETAL HEART. RNA was extracted and subjected to Northern analysis, as described in 5.2.5. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA, stripping the bound probe in boiling water in between hybridisations. Representative gels from a subset of animals are shown. Lane 1 = normal adult ewe heart; Lane 2 = 30 days gestation; Lane 3 = 60 days; Lane 4 = 95 days; Lane 5 = 120 days; Lanes 6 and 7 = 130 days; Lanes 8 and 9 = 145 days ; Lanes 10 and Lane 11 = 1 day post-natal lamb; Lane 12 = normal adult ewe liver (control) .

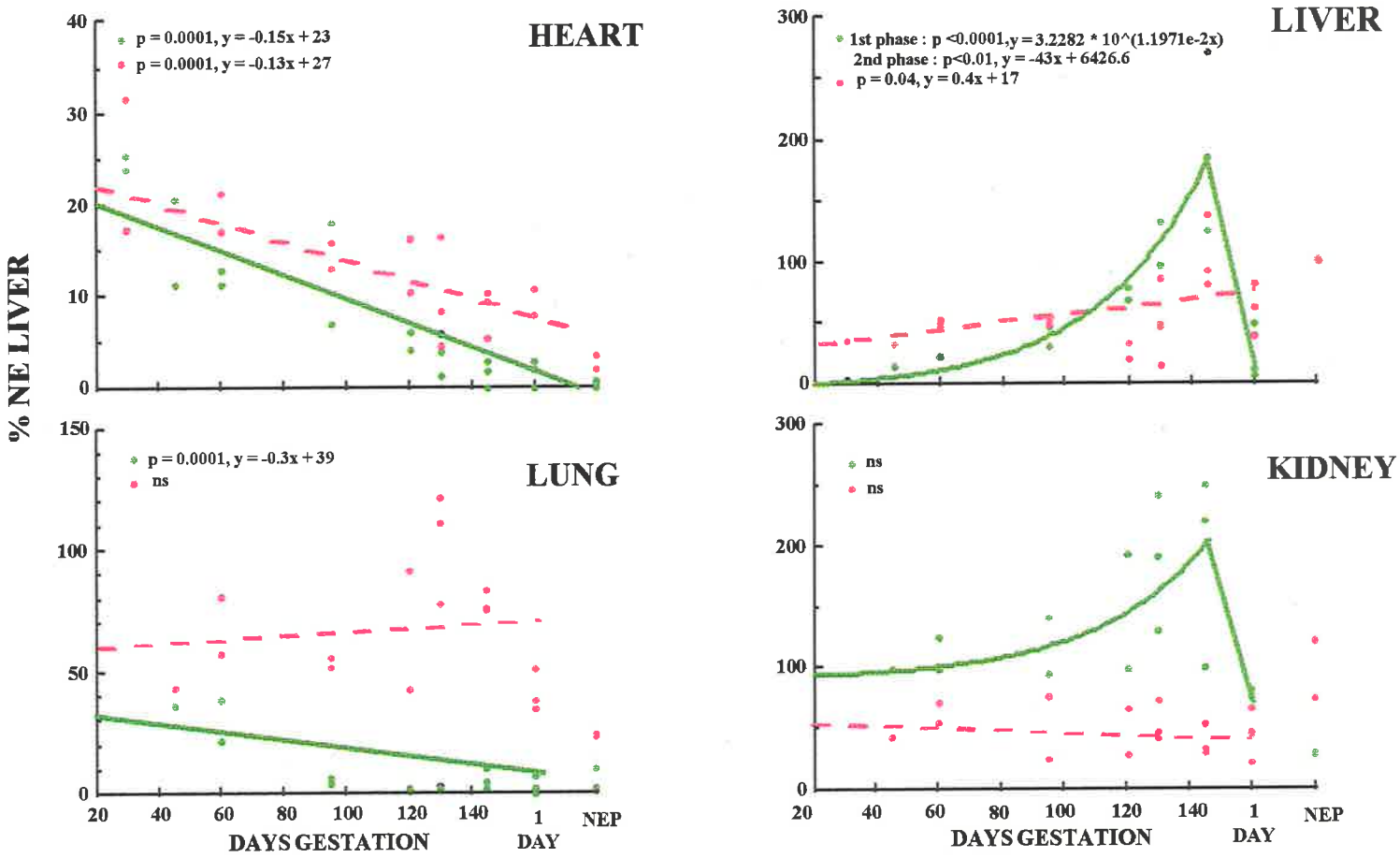


FIGURE 5.10 QUANTITATION OF TISSUE mRNA LEVELS OF IGFBP-2 AND IGFBP-4. Autoradiographs from Northern blots for IGFBP-2, IGFBP-4 and 18S rRNA were scanned by laser densitometry, values expressed as a percentage of 18S rRNA then normalised against the adult ewe liver control on each gel. Data was analysed by linear or quadratic regression analysis. Liver IGFBP-2 mRNA was analysed with a segmented model. P values and regression lines are indicated. ns = not significant. IGFBP-2 (●) = solid green lines. IGFBP-4 (●) = dashed red lines.

5.3.4 CIRCULATING IGFs IN THE OVINE FETUS

To assess the relationships between IGFBPs and circulating IGFs, both IGF-I and IGF-II were quantitated by acid-gel chromatography followed by RIA. Initially, elution profiles of IGF-I and IGF-II immunoreactivity in fetal plasma at 60 and 140 days gestation, 1 day lamb and normal ewe were established (Figure 5.11). IGF-I and IGF-II immunoreactivity eluted in two peaks. However, for IGF-II, peak II shows a higher molecular weight shoulder in all samples except the normal ewe (Figure 5.11). Western ligand blotting indicates peak I to represent IGFBPs, while peak II, or the shoulder to peak II does not contain IGFBPs detectable by this technique (Figure 5.12). Fractions from peak I, peak II and the shoulder to peak II were incubated with ^{125}I-IGF-II and subjected to neutral gel filtration to assess IGF binding activity (Figure 5.13). Peak I, but not peak II or the shoulder region, shifted the elution of ^{125}I-IGF-I to a higher molecular weight species, indicating the presence of IGF-binding activity in peak I only. Individual assay of fractions from 7.75 to 10 minutes in all samples, showed that this shoulder of IGF-II immunoreactivity was negligible compared to the major IGF-II peak (data not shown). Therefore, immunoreactivity in fractions 8.5 to 10 minutes were summed to obtain total IGF-II. The same fractions were summed to quantitate total IGF-I. Circulating IGF-I increased progressively from 60 days through to 1 day post-natally ($p = 0.0001$, Figure 5.14). IGF-II increased between 60 and approximately 105 days gestation then decreased ($p = 0.01$, Figure 5.14).

5.3.5 CORRELATION ANALYSIS

Tables 5.1 and 5.2 summarise the major correlations between circulating IGFBPs, IGFs, IGFBP mRNA, fetal weight *in utero* or at all ages. Circulating IGFBP-2 is positively correlated with fetal weight *in utero*, liver IGFBP-2 mRNA and kidney IGFBP-2 mRNA. Circulating IGFBP-3 (measured by RIA or Western ligand blotting) correlated with fetal weight and circulating IGFBP-4. Immunoreactive IGFBP-3 correlated positively with IGF-I and IGF-II *in utero*. Circulating IGFBP-4 is correlated with fetal weight, liver IGFBP-4 mRNA, circulating IGFBP-3 (measured by RIA or Western ligand blotting) and IGF-I.

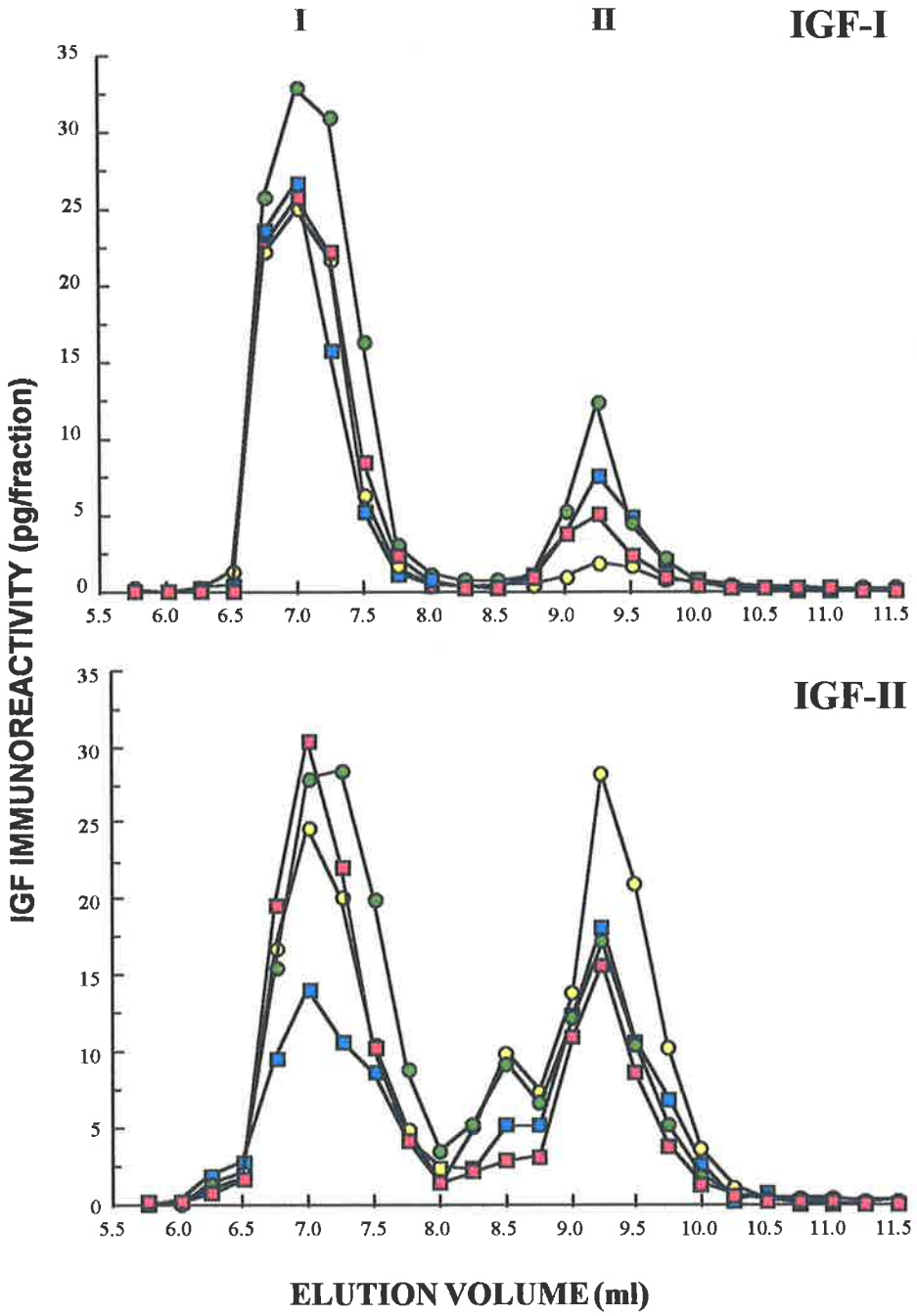
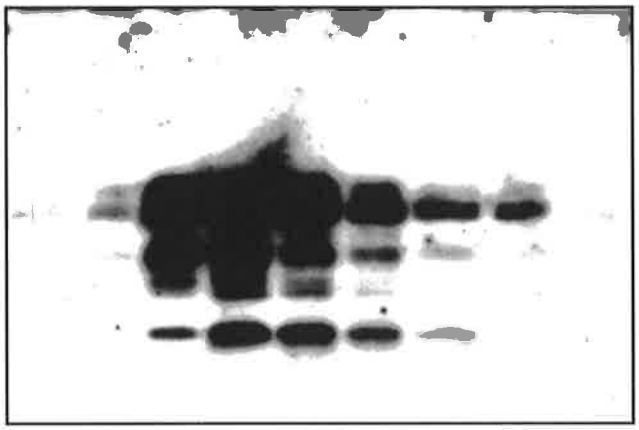
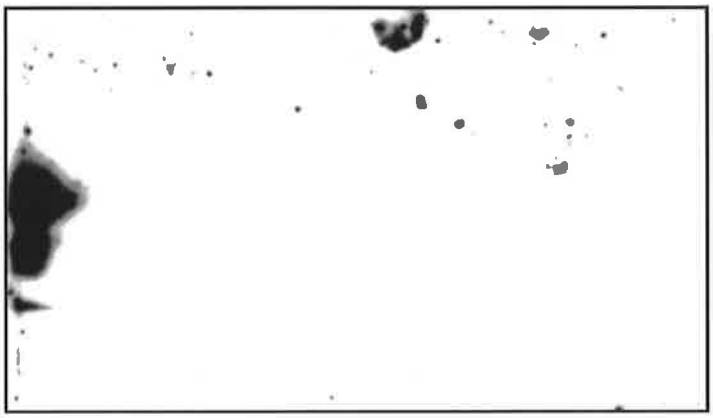


FIGURE 5.11 IMMUNOREACTIVITY ELUTION PROFILES OF ACID-GEL CHROMATOGRAPHED OVINE PLASMA. Plasma was acidified and chromatographed and fractions subjected to IGF-I or IGF-II RIA, as described in 5.2.6. ○ = 60 days gestation; ■ = 145 days gestation; ● = 1 day post-natal lamb; ■ = normal adult ewe plasma. Peaks I and II are indicated.



1 2 3 4 5 6 7 8 9



19 18 17 16 15 14 13 12 11 10

LANE NUMBER

FIGURE 5.12 WESTERN LIGAND BLOT OF FRACTIONS FROM ACID-GEL CHROMATOGRAPHED OVINE PLASMA. Plasma from a 60 day gestation ovine fetus was subjected to acid-gel chromatography, as described in 5.2.6. Fractions were concentrated, neutralised and subjected to Western ligand blotting, as described in 5.2.3. Lanes 1 to 18 represent 0.25 ml fractions, elution volume 6.25 to 10.5 ml, respectively. Lane 19 = normal adult ewe plasma (2 µl).

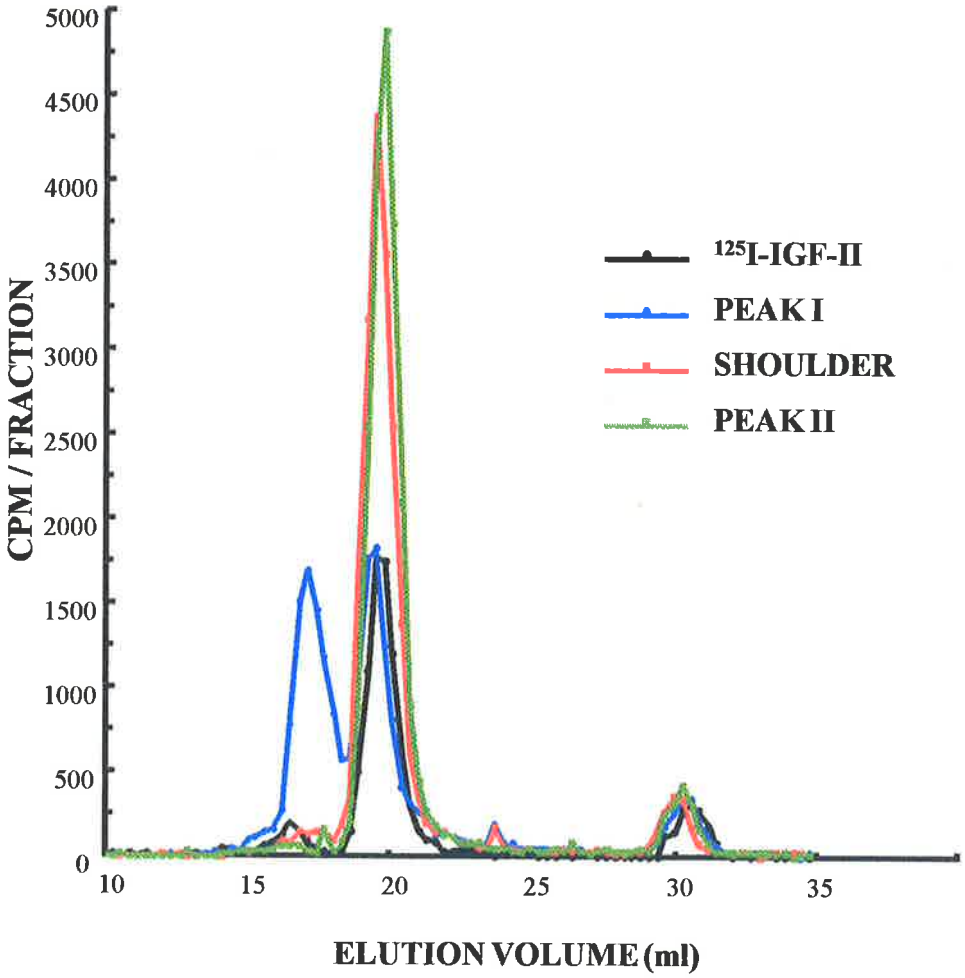


FIGURE 5.13 ^{125}I-IGF-II BINDING ACTIVITY OF FRACTIONS FROM ACID-GEL CHROMATOGRAPHY OF OVINE PLASMA. Ovine plasma from a 1 day post-natal lamb was subjected to acid-gel chromatography, as described in 5.2.6. Fractions from 6.5 to 7.75 (peak I), 8.25 to 8.5 (shoulder) and 8.75 to 10 ml elution volume (peak II) were pooled and 3 ng equivalents of IGF-II immunoreactivity incubated ^{125}I-IGF-II (5.2.7). Reactions were subjected to neutral gel filtration and fractions counted. Approximately 5,000 cpm of ^{125}I-IGF-II was chromatographed alone as a control.

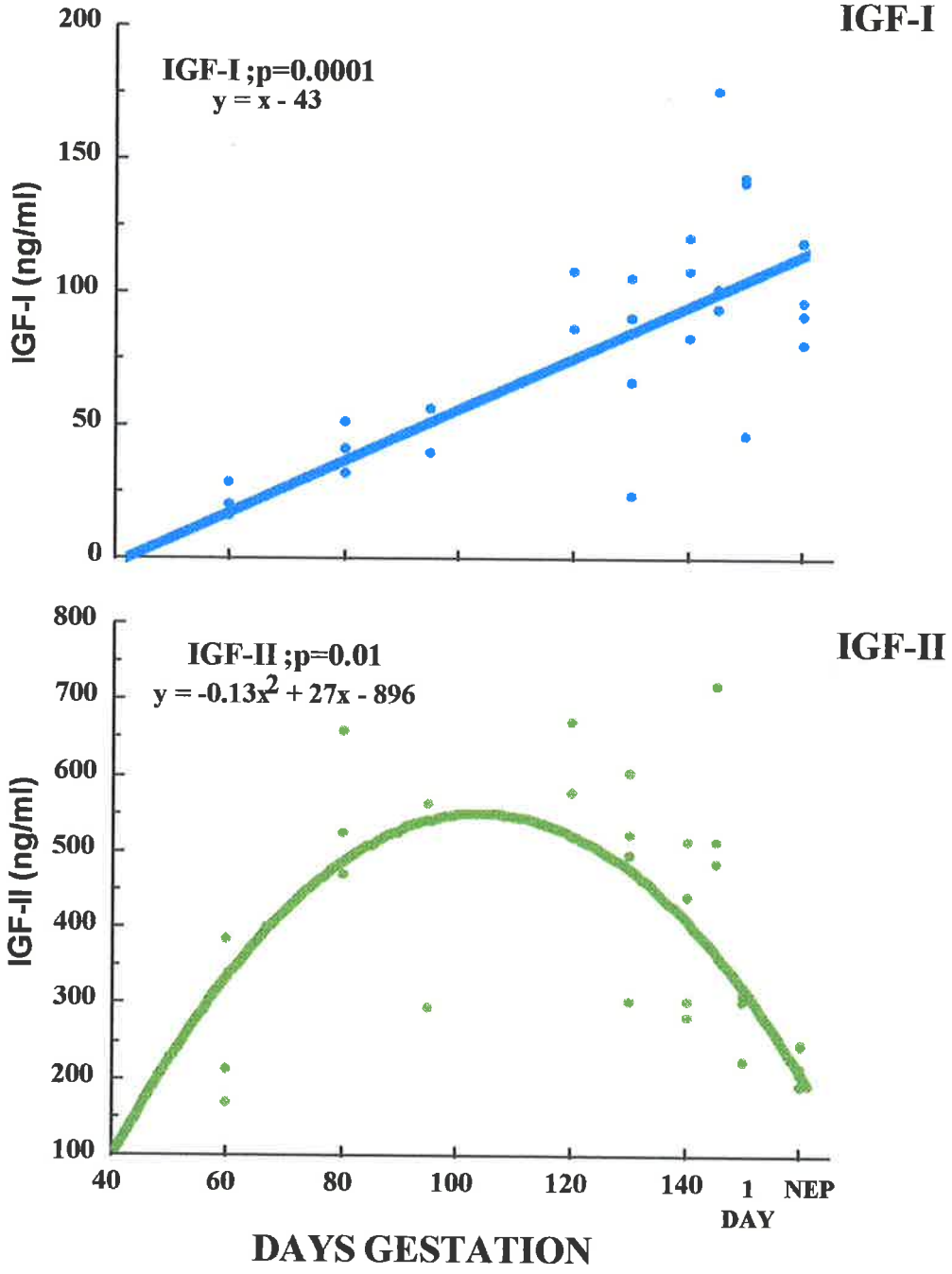


FIGURE 5.14 QUANTITATION OF CIRCULATING IGF-I AND IGF-II BY RIA. Plasma was acid-gel chromatographed and fractions subjected to IGF-I and IGF-II RIA, as described in 5.2.6. Samples were as follows : 60 days, n = 3; 80 days, n = 3; 95 days, n = 2; 120 days, n = 3; 130 days, n = 4; 140 days, n = 4; 145 days, n = 3; 1 day post-natal lamb, n = 3. Data was analysed using linear or quadratic regression models. Regression lines and p values are indicated.

		FETAL WEIGHT	IGF-I	IGF-II	IGFBP-2	IGFBP-3 (RIA)	IGFBP-3 (WLB)	IGFBP-4
FETAL WEIGHT	r p	-	0.66 0.027	ns	0.67 0.003	0.59 0.03	0.58 0.01	0.62 0.007
IGF-I	r p	0.66 0.027	-	0.53 *ns* 0.01	ns *ns*	0.54 *0.61* 0.01 *0.002*	ns *ns*	0.52 *0.44* 0.01 *0.03*
IGF-II	r p	ns	0.53 *ns* 0.01	-	*0.37 *0.48* *0.09 *0.02*	0.43 *ns* 0.05	ns *ns*	ns *ns*
IGFBP-2	r p	0.67 0.003	ns *ns*	*0.37 *0.48* *0.09 *0.02*	-	ns *ns*	0.31 *ns* 0.05	ns *ns*
IGFBP-3 (RIA)	r p	0.59 0.03	0.54 *0.61* 0.01 *0.002*	0.43 *ns* 0.05	ns *ns*	-	0.55 *0.49* 0.0006 *0.001*	0.50 *0.44* 0.002 *0.003*
IGFBP-3 (WLB)	r p	0.58 0.01	ns *ns*	ns *ns*	0.31 *ns* 0.05	0.55 *0.49* 0.0006 *0.001*	-	0.33 *0.32* 0.04 *0.03*
IGFBP-4	r p	0.62 0.007	0.52 *0.44* 0.01 *0.03*	ns *ns*	ns *ns*	0.50 *0.44* 0.002 *0.003*	0.33 *0.32* 0.04 *0.03*	-

TABLE 5.1 RELATIONSHIPS BETWEEN CIRCULATING FACTORS AND FETAL WEIGHT. Correlation analysis was performed as described in 5.2.8, analysing data *in utero* seperately or with 1 day post-natal values. r = Pearsons correlation co-efficient, p = probability. Values in plain text indicate correlations *in utero*. Values in italics indicate correlations throughout gestation and 1 day post-natally. * = correlation approaches significance

		IGF-I	IGF-II	IGFBP-2	IGFBP-4	HEART -2 mRNA	LIVER -2 mRNA	LUNG -2 mRNA	KIDNEY -2 mRNA	HEART -4 mRNA	LIVER -4 mRNA
HEART -2 mRNA	r	-0.92	ns	-0.82	ns	-	-0.80	0.87	-0.84	0.77	ns
	p	0.03		0.03			0.02	0.005	0.009	0.05	
LIVER -2 mRNA	r	ns	ns *0.65*	0.77 *0.82*	0.79 *0.74*	-0.80	-	ns	0.73 *0.76*	-0.86	0.86
	p		*0.04*	0.005 *0.0003*	0.004 *0.002*	0.02			0.005 *0.0006*	0.01	0.0002
LUNG -2 mRNA	r	-0.95	ns	-0.84	ns	0.87	ns	-	-0.80	ns	ns
	p	0.004		0.008		0.005			0.009		
KIDNEY -2 mRNA	r	ns	ns	0.72 *0.80*	0.72 *0.65*	-0.84	0.73 *0.76*	-0.80	-	-0.79	ns
	p			0.01 *0.0007*	0.01 *0.01*	0.009	0.005 *0.0006*	0.009		0.034	
HEART -4 mRNA	r	ns	ns	-0.86	-0.91	0.77	-0.86	ns	-0.79	-	-0.80
	p			0.03	0.01	0.05	0.01		0.034		0.031
LIVER -4 mRNA	r	ns	ns	ns	0.73	ns	0.86	ns	ns	-0.80	-
	p				0.01		0.0002			0.031	

TABLE 5.2 RELATIONSHIPS BETWEEN CIRCULATING FACTORS AND IGFBP-2 and IGFBP-4 mRNA. Correlation analysis was performed as described in 5.2.8, analysing data *in utero* seperately or with 1 day post-natal values. r = Pearsons correlation co-efficient, p = probability. Values in plain text indicate correlations *in utero*. Values in italics indicate correlations throughout gestation and 1 day post-natally.

IGF-I is positively correlated with fetal weight, circulating IGFBP-4, IGFBP-3 (measured by RIA) and IGF-II *in utero*, and negatively correlated with heart IGFBP-2 and IGFBP-4 mRNA. When analysed *in utero* only, IGF-II correlated with IGF-I and IGFBP-3. With data from the 1 day neonatal lambs included, circulating IGF-II positively correlated with circulating IGFBP-2 and liver IGFBP-2 mRNA. IGFBP-2 and IGFBP-4 mRNAs are positively correlated in the liver and heart, but not in the lung or kidney.

5.4 DISCUSSION

The current study has defined the major changes in circulating IGFBPs throughout gestation in the ovine fetus. IGFBP-2 levels rose to peak at approximately 120 days gestation then declined by 1 day post-natally. These changes in circulating IGFBP-2 are similar to IGFBP-2 levels measured by RIA in the fetal pig (McCusker *et al.*, 1991), although our results represent a much larger number of observations across a wider time frame of gestation. In contrast to the changes observed in IGFBP-2, IGFBP-3 levels rose steadily from 45 days gestation to 1 day post-natally, as do IGFBP-4 levels. Circulating IGFBP-3 was measured by Western ligand blotting and by RIA, the latter being a more precise assay of IGFBP protein levels. Measurement of IGFBPs by Western ligand blot is a functional indication of IGF binding activity and may only reflect circulating IGFBP protein levels in the absence of substantial post-translational modifications, that can alter IGF binding affinity under Western ligand blot conditions. Post-translational modifications of IGFBPs can not be excluded in this study. However, the positive correlation between IGFBP-3 measured by Western ligand blot or RIA suggest at least IGFBP-3 measured by Western ligand blot closely reflects circulating IGFBP-3 levels.

The ontogenic changes in circulating IGFBP-2 and IGFBP-4 parallel and were correlated with their respective steady state mRNA levels in the liver. Circulating IGFBP-2 also correlated with kidney IGFBP-2 mRNA. Of the four tissues studied, only the fetal liver and kidney produce significant amounts of IGFBP-2 mRNA in late gestation. In contrast, IGFBP-2 mRNA is low in the heart or lung of fetuses older than 60 days gestation. Thus, the liver and kidney may be the primary contributors to circulating IGFBP-2. Consistent

with this, IGFBP-2 mRNA has previously been reported to be present in all tissues of the fetal sheep, when examined at 50 to 75 days gestation, but restricted to only the liver, kidney and choroid plexus at later gestational ages (80 to 145 days) (Delhanty and Han, 1993). It was also observed that of the four tissues examined at any fetal age, the highest levels of IGFBP-2 mRNA were found in the fetal kidney, as has previously been reported (Delhanty and Han, 1993). Consistent with this earlier study, it was also found in this study that liver IGFBP-2 mRNA increased with advancing gestational age (Delhanty and Han, 1993). However, a decrease in liver IGFBP-2 mRNA was observed in this study between late gestation and 1 day post-natally, in contrast to previous reports of a continued increase up to 1 to 60 days post-natally (Delhanty and Han, 1993). A decline in kidney IGFBP-2 mRNA was also found to occur between approximately 145 days and 1 day post-natally, rather than between 120 to 145 days gestation and 1 to 60 days post-natally, as suggested previously (Delhanty and Han, 1993). These differences may arise from the wide range of ages (120 to 145 days gestation) at which tissues were analysed previously (Delhanty and Han, 1993). The grouping of fetuses at wide gestation ages in this previous study, may have obscured the major changes in fetal kidney and liver IGFBP-2 mRNA that the current study has shown to occur during the transition from the intra-uterine environment to post-natal life .

Circulating IGFBP-4 positively correlated with liver IGFBP-4 mRNA, suggesting that the liver may be the major source of circulating IGFBP-4 in the fetus, although the kidney and lung continue to produce high levels of IGFBP-4 mRNA throughout gestation. IGFBP-4 mRNA is low in the heart at later gestational ages, as observed for IGFBP-2 mRNA. IGFBP-2 and IGFBP-4 mRNA abundance correlated in the heart and liver, but not in the lung or kidney, suggesting that in the former tissues regulation of IGFBP-2 and IGFBP-4 mRNA may be co-ordinate, but in the latter, that these IGFBPs may be controlled independently.

In many physiological situations, IGFBP levels are highly positively correlated with those of IGFs (Cohick and Clemmons, 1993). In young growing pigs, the developmental increase in plasma IGF-I and IGF-II is strongly associated with an increase in circulating

IGFBP activity (Owens *et al.*, 1991). Similarly, the sum of circulating IGF-I and IGF-II is strongly correlated with IGFBP-3 in man (Baxter, 1988). In this chapter, the circulating IGFs in the ovine fetus have been characterised, over a greater part of gestation than described before and show an increase in IGF-I throughout gestation, as indicated previously (D'Ercole *et. al.*, 1980; Moses *et al.*, 1980; Gluckman and Butler, 1983; Mesiano *et. al.*, 1989). Plasma IGF-II concentrations increased in early gestation, are at high levels throughout mid gestation then declined over late gestation. In combination with the circulating changes shown in IGFBPs, these results show that circulating IGF-II and IGFBP-2 are high in the fetus in mid to late gestation and decrease near term towards adult levels, while circulating IGF-I and IGFBP-3 are lower in early to mid gestation and increase throughout gestation to reach adult values. This is consistent with gel filtration data in the sheep (Butler and Gluckman, 1986) and observations in the pig and primate (Liu *et al.*, 1991; Wang and Chard, 1992; Lee *et al.*, 1993a). Acid-gel chromatography of fetal plasma revealed an abnormal shoulder of IGF-II immunoreactivity in fetal and 1 day lamb plasma that is not present in adult ewes and does not represent IGF-binding activity. Similar higher molecular weight immunoreactivity has been previously observed in plasma from tumour bearing patients (K. J. Quinn and P. C. Owens, pers. comm.) and may represent unprocessed or big IGF-II (Daughaday and Rotwein, 1989). However, in contrast to the observations in tumour bearing patients, the amount of this immunoreactivity in the ovine fetus was minor compared to the total amount of circulating IGF-II.

Circulating IGF-I, but not IGF-II, was shown to positively correlate with fetal weight, consistent with circulating IGF-I influencing or reflecting fetal growth, whereas circulating IGF-II may reflect or determine additional factors or processes within the fetus. The levels of circulating IGF-I positively correlated with circulating IGFBP-3 and IGFBP-4, while circulating IGF-II was positively correlated with circulating IGFBP-2 and liver IGFBP-2 mRNA. These associations indicate a linkage of specific IGFs with IGFBPs. They also suggest that IGF-I or IGF-II may regulate specific IGFBPs or vice versa. Alternatively, IGFs and IGFBPs may be co-regulated by common factors.

The decline in circulating IGF-II, IGFBP-2 and liver IGFBP-2 mRNA in the fetal

sheep, just before term, observed in this study parallels that seen in IGF-II mRNA in the ovine fetal liver in late gestation (O'Mahoney *et al.*, 1991; Li *et al,* 1993). Furthermore, liver IGF-II mRNA, between 140 and 145 days gestation in the ovine fetus correlates negatively with circulating fetal cortisol levels (Li *et al.*, 1993). IGF-II mRNA is also negatively related to glucocorticoids in the neonatal rat (Beck *et al.*, 1988; Levinovitz and Norstedt, 1989). Thus, cortisol inhibition of IGF-II mRNA levels has been suggested partly responsible for the observed ontogeny of IGF-II mRNA and protein. Thus it can be hypothesised that IGF-II and IGFBP-2 liver mRNA may be regulated directly by a common factor, such as cortisol, or that cortisol may alter IGF-II production that in turn influences IGFBP-2.

In conclusion, results described in this chapter have defined the ontogenic changes in circulating IGFs and IGFBPs throughout fetal development and at parturition. The ontogeny of IGFBP-2 and IGFBP-4 mRNA in major fetal tissues throughout gestation have been characterised and show strong developmental and tissue-specific regulation of IGFBP mRNA levels. Although regulation of IGFBP mRNA is likely to be multifactorial, possible candidates that have been implicated as regulatory factors of IGFBP production in the fetus, include IGF-I, IGF-II and cortisol.

CHAPTER 6. CIRCULATING IGFBPs AND TISSUE mRNA FOR IGFBP-2 AND -4 IN THE GROWTH RETARDED OVINE FETUS

6.1 INTRODUCTION

The IGFs have been shown to be important for fetal growth and development by an abundance of indirect evidence and most recently, directly by gene knock-out experiments (1.3.2). Strong developmental and tissue specific patterns of expression of IGFBPs throughout gestation in the ovine fetus have been demonstrated (Chapter 5), which together with previous studies (1.3.3) suggest important functions for IGFBPs in normal growth and development of the fetus. This study aimed to examine the ontogeny of the IGFBP axis in a situation where growth and development of the fetus was perturbed. The perturbation used in this study was experimental induction of intra-uterine growth retardation (IUGR), by restriction of placental implantation and development, and hence restriction of substrate supply to the ovine fetus (Robinson *et al.*, 1979). Between 70 to 90 % of the variation in birthweight can be explained by that in placental weight, indicating the substantial influence of the placenta on fetal growth (Mellor, 1983). The sheep placenta is cotyledonary in nature, being comprised of between 50 to 100 individual structures, called cotyledons, or placentomes. These are formed by the fusion of the invading blastocyst with maternal endometrial attachment sites, termed caruncles, which in the ovine placenta forms the sites for nutrient and gas exchange between the mother and the fetus (Hay and Wilkening, 1994). Restriction of placental growth and substrate transfer function can be achieved through surgical removal of most maternal endometrial caruncles, or implantation sites, prior to pregnancy. The reduction in the number of sites available for implantation results in reduced numbers of cotyledons, total placental weight and fetal weight, in late gestation (Robinson *et al.*, 1979). The growth retardation produced is asymmetric, with conservation of particular tissues in terms of weight, such as the brain, but proportional reduction in weights of other organs, such as the liver, heart and lung, to a similar or greater extent than body weight (Robinson *et al.*, 1979). However, there are also major structural alterations in

many organs, including that of the brain, which on the basis of weight, may appear unaffected by growth restriction (Robinson *et al.*, 1994). The placenta is also altered by restriction of implantation, with a re-distribution of placentome size towards a higher mean cotyledon weight and alterations in placental morphology which are associated with compensatory structural changes in the cotyledons (Chidzanja, 1994). Wool development is also markedly retarded (Harding *et al.*, 1985). The metabolic and endocrine state of the growth restricted fetus is altered, with the animals becoming hypoxaemic and hypercapnic, with decreases in blood glucose and insulin levels and increases in circulating cortisol and catecholamines (Robinson *et al.*, 1994). Both circulating fetal IGF-I and IGF-II decrease late in gestation with severe placental restriction of fetal growth (Owens *et al.*, 1994). However, circulating fetal IGF-II levels are unchanged or increased in the moderately restricted ovine fetus in the early stages of late gestation (Jones *et al.*, 1988; Owens *et al.*, 1994). Since fetal growth retardation is associated with alterations in the abundance of IGFs and metabolic and endocrine changes, that in post-natal life would alter IGFBPs, alterations in the IGFBP axes may also occur in the growth restricted ovine fetus. The abundance of IGFs and IGFBPs are altered in experimental IUGR in the rodent, induced by procedures such as uterine artery ligation, maternal fasting in late gestation, treatment with dexamethasone or more recently by maternal hypoxia (1.3.4) (Price *et al.*, 1992; Donovan *et al.*, 1991; Straus *et al.*, 1991; Unterman *et al.*, 1993a and 1993b; Tapanainen *et al.*, 1994). These studies show that acute restriction of growth in the fetal rodent in late gestation, produced by a variety of insults, is consistently associated with reduced circulating IGF-I and increased IGFBP-1. In contrast, IGF-II has been reported to either increase, decrease or remain unchanged, while IGFBP-2 is either elevated or unchanged when fetal growth is restricted in the rodent (Price *et al.*, 1992; Donovan *et al.*, 1991; Straus *et al.*, 1991; Unterman *et al.*, 1993a and 1993b; Tapanainen *et al.*, 1994). Experimental restriction of fetal growth in the sheep induced by placental restriction of implantation mimics the most common type of restriction experienced naturally, in larger mammalian species, where the fetal IGF / IGFBP axes are similar to other mammals, such as the human and the pig, but differs from that in the rodent (Chapter 1, Table 1.4). In

addition the size and tolerance of the sheep allows chronic experimentation and endocrine measurements in the absence of the stresses associated with anaesthesia. Thus, the model of growth restriction described in this study differs from the previously described rodent models of fetal growth restriction.

In this study the ontogeny of circulating IGFBP-2, -3 and -4 has been characterised in the growth retarded sheep fetus throughout mid to late gestation. Additionally, the tissue distribution and abundance of IGFBP-2 and IGFBP-4 mRNA were analysed in the heart, liver, lung and kidney in a subset of these animals. Results indicate that IGFBPs in the growth restricted fetus are altered in a manner which varies with the particular IGFBP and with gestational age, such that circulating IGFBP-2 is elevated in mid gestation and circulating IGFBP-4 in late gestation, in the growth restricted fetus. The tissue distribution and relative abundance of IGFBP-2 and IGFBP-4 mRNA, in the heart, liver, lung or kidney, was unchanged by restriction of fetal growth. However, additional animals should be studied to accurately determine if restriction of fetal growth results in quantitative changes in the mRNA abundance for IGFBP-2 or IGFBP-4 in fetal tissues, that may contribute to changes in circulating proteins. These developmental specific changes in circulating IGFBP levels, in combination with alterations in IGF abundance, may contribute to the overall restriction and pattern of altered fetal growth resulting from restriction of placental implantation and development.

6.2 MATERIALS AND METHODS

6.2.1 BLOOD AND TISSUE COLLECTION

Fetal growth retardation was experimentally produced by maternal caruncleclectomy as described by Robinson *et al.* (1979). Briefly, most endometrial placental implantation sites were surgically removed from the uteri of ewes prior to pregnancy. After recovery from surgery (at least 6 weeks), the ewes were mated. Catheters were inserted into the fetal femoral artery and umbilical vein, to allow for serial blood sampling, as described previously (Robinson *et al.*, 1979). Post-mortem analysis was carried out at the desired gestational ages, to determine fetal organ and body weights, placental weights and cotyledon numbers.

Blood samples were collected prior to post-mortem for IGFBP analysis. All surgical techniques, blood sampling and post-mortems were performed by co-workers at the Departments of Obstetrics and Gynaecology and of Physiology, University of Adelaide, South Australia. Gestational ages were as follows 95 days (n = 4), 120 days (n = 9), 140 days (n = 6). Tissues were snap frozen in liquid nitrogen for subsequent RNA extraction at 95 days (n = 1) and 120 days (n = 3). In addition, blood (n = 6) and tissues (n = 3) were obtained from fetuses subjected to placental restriction of substrate supply and intra-uterine growth retardation, as a result of twinning.

6.2.2 WESTERN LIGAND BLOTTING AND NORTHERN ANALYSIS OF IGFBP-2 AND IGFBP-4 mRNA

Western ligand blotting and Northern analysis of IGFBP-2, IGFBP-4 and 18S rRNA was performed and quantitated, as described in Chapters 2 and 5.

6.2.3 FETAL BLOOD SUBSTRATE AND GAS ANALYSES

Fetal umbilical vein pO_2, pCO_2 and pH were measured using a Radiometer ABL 330 Blood gas Analyser (Radiometer, Copenhagen). Haemoglobin concentration and fetal umbilical vein oxygen saturation were determined using a Radiometer OSM2 Hemoximeter (Radiometer, Copenhagen). Blood glucose levels were measured in some fetuses at 120 and 140 days gestation and insulin concentrations were determined in fetuses at 120 and 130 days gestation (Owens *et al.*, 1994). Circulating IGF-I and IGF-II levels were determined as described by Owens *et al.* (1994) at 120 days gestation. These IGF measurements differ to the procedure described in Chapter 5 due to the use of a different IGF-I antibody and an IGF-II radioreceptor assay, rather than the IGF-II RIA used in Chapter 5, although both assays gave similar results. All substrate and gas analyses were performed by co-workers at the Departments of Obstetrics and Gynaecology and of Physiology, University of Adelaide, Adelaide, South Australia.

6.2.4 STATISTICAL ANALYSIS

The effects of restriction of placental growth on parameters was examined by one way analysis of variance (ANOVA) (Proc GLM, SAS, SAS Institute Inc. Cary, NC, USA) with specific contrasts by least squares means. Relationships between variables were examined by correlation analysis (Proc Corr, SAS, SAS Institute Inc. Cary, NC, USA).

6.3 RESULTS

6.3.1 THE EFFECT OF RESTRICTED PLACENTAL IMPLANTATION ON FETAL GROWTH AND METABOLISM

Figure 6.1 shows fetal and placental weights of animals subjected to placental restriction of implantation. Fetal weight increased and placental weight decreased with advancing gestational age ($p = 0.0001$ and $p = 0.0008$, respectively). Restriction of placental implantation reduced placental weight from 120 days gestation onwards ($p < 0.05$). Restriction of placental implantation also reduced fetal weight over this same period of gestation ($p = 0.0001$, at all ages). Mean values at each fetal age for fetal organ and body weights, umbilical vein oxygen saturation, pO_2, p CO_2 and blood pH from both growth restricted and normal fetuses, are shown in Table 6.1, except for 140 days gestation, where arterial measures are shown. Placental restriction reduced fetal blood pO_2 and oxygen saturation (Table 6.1). The weights of major fetal organs, with the exception of the brain, were reduced in fetuses subjected to restricted placental implantation (Table 6.1). Table 6.2 also shows fetal organ weights expressed as a percentage of body weight, indicating the asymmetry of growth, with fetal organ to body weight ratios decreased or unchanged in the placenta, liver, kidney, lung and heart, but increased in the brain. Thus restriction of placental implantation and development reduces placental weight, fetal body weight, some organ weights and circulating substrate levels in the fetus.

6.3.2 CIRCULATING IGFBPs IN THE GROWTH RESTRICTED FETUS

Circulating IGFBPs in normal and growth retarded fetuses were analysed by Western ligand blotting. Figure 6.2 shows a Western ligand blot of representative plasma

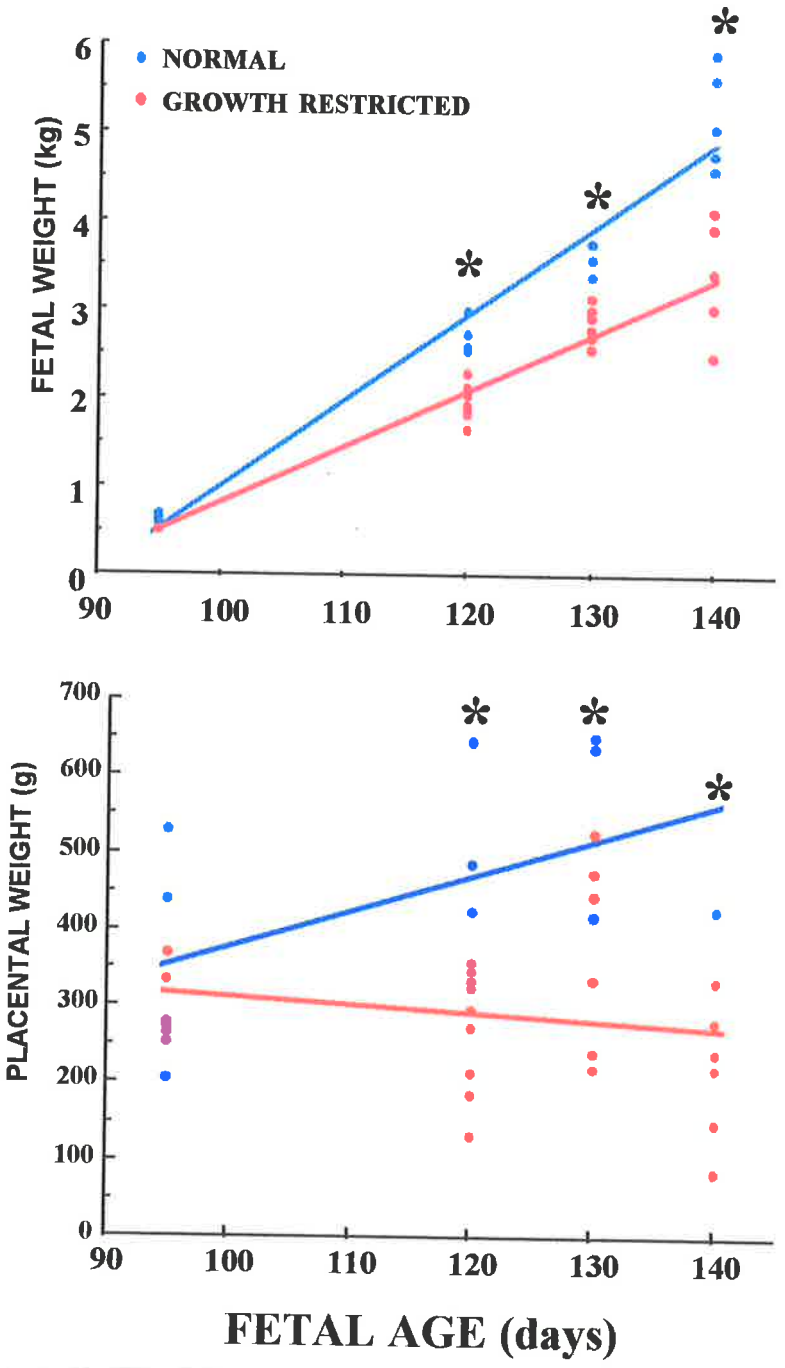


FIGURE 6.1 FETAL AND PLACENTAL WEIGHTS OF NORMAL AND GROWTH RESTRICTED FETUSES. Weights were determined at post-mortem at 95, 120, 130 and 140 days gestation and compared to normal fetuses (Chapter 5). ANOVA indicates fetal and placental weights varied with treatment and age, with increasing fetal weight and decreasing placental weight with age in growth restricted fetuses ($p = 0.0001$ and $p = 0.008$, respectively). Specific comparisons, by least squares means, shows that fetal and placental weights were reduced from 120 to 140 days gestation, indicated by *. Lines of best fit (Cricket Software) are indicated (formula and r^2 values not shown). Normal placental weights at 140 days represent the mean value obtained from a previous study (J. A. Owens, pers. comm.).

GESTATIONAL AGE		95 DAYS	120 DAYS	130 DAYS	140 DAYS
COTYLEDON NUMBER	Normal	71.2 ±4.1	67.3 ± 4.6	68 ± 5	NA
	Restricted	26.3 ± 4.6 *	28.9 ± 3.2 *	45 ± 3.7 *	NA
PLACENTAL WEIGHT (g)	Normal	346 ± 48	473 ± 54	570 ± 62	430 ± 28
	Restricted	306 ± 54	265 ± 38 *	374 ± 44 *	220 ± 44 *
FETAL WEIGHT (kg)	Normal	0.65 ± 0.20	2.61 ± 0.22	3.57 ± 0.25	5.50 ± 0.3
	Restricted	0.56 ± 0.22	1.99 ± 0.16 *	2.86 ± 0.18 *	3.35 ± 0.18 *
BRAIN WEIGHT (g)	Normal	16.9 ± 12.2	40.1 ± 13.7	50.9 ± 13.7	NA
	Restricted	18.03 ± 0.34	40.6 ± 9.7	48.1 ± 11.2	NA
LIVER WEIGHT (g)	Normal	47 ± 9.3	116.9 ± 10.4	138 ± 10.4	149 ± 15
	Restricted	30.4 ± 10.4	73.8 ± 7.4 *	91.3 ± 8.5 *	66.4 ± 8.5 *
KIDNEY WEIGHT (g)	Normal	3.9 ± 0.7	10.6 ± 0.7	13.1 ± 0.7	13.5 ± 0.7
	Restricted	3.4 ± 0.7	7.5 ± 0.5 *	10.1 ± 0.6 *	9.1 ± 0.6 *
HEART WEIGHT (g)	Normal	5.9 ± 1.2	19.2 ± 1.3	26.6 ± 1.3	37.6 ± 2.7
	Restricted	4.9 ± 1.3	15.5 ± 0.9 *	22.9 ± 1.1 *	23.9 ± 1.1 *
LUNG WEIGHT (g)	Normal	17.5 ± 6.5	75.5 ± 7.3	102 ± 7	174 ± 11
	Restricted	18 ± 7	71 ± 5	88 ± 6	82 ± 6 *
BLOOD pH	Normal	7.32 ± 0.01	7.36 ± 0.02	7.36 ± 0.02	*7.38 ± 0.08*
	Restricted	7.34 ± 0.02	7.35 ± 0.01	7.37 ± 0.02	*7.37 ± 0.01*
BLOOD pO_2 (mm Hg)	Normal	30.9 ± 3.4	30.7 ± 3.8	32.4 ± 3.8	*20.3 ± 1.9*
	Restricted	34.6 ± 3.8	25.3 ± 2.7	28.5 ± 3.5	*14.2 ± 3.1*
% O_2 SATURATION	Normal	78.5 ± 6.3	79.1 ± 7.1	76.8 ± 7.1	*59.1 ± 6.2*
	Restricted	76.6 ± 7.1	73.9 ± 5	74.3 ± 7.1	*43.2 ± 5.8*
BLOOD pCO_2 (mm Hg)	Normal	48.7 ± 2.3	50.2 ± 2.6	42.5 ± 2.6	*47.7 ± 2.9*
	Restricted	54.2 ± 2.6	50.1 ± 1.8	47.7 ± 2.6	*46.9 ± 2.1*

TABLE 6.1 FETAL WEIGHTS AND CIRCULATING SUBSTRATE LEVELS. Mean values +/- SEM (standard error of the mean) are shown for normal fetuses and fetuses subjected to restriction of placental implantation. * indicates significantly different to controls by least squares means ($p<0.05$). Italics indicates measurements from the fetal femoral artery. NA = data not obtained. For the normal fetuses n = 5, 95 days; n = 4, 120 days; n = 4, 130 days; n = 8, 140 days, except for placental weights, n = 9, 140 days, obtained from a concomitant study by J. A. Owens. For growth restricted fetuses n = 4, 95 days; n = 8 120 days; n = 6, 130 days and n = 6, 140 days.

		95 DAYS	120 DAYS	130 DAYS	140 DAYS
PLACENTAL : BODY WEIGHT (%)	Normal	53.2	181	159	78
	Restricted	54.6	133	130	65
BRAIN : BODY WEIGHT (%)	Normal	2.6	1.5	1.4	NA
	Restricted	3.2	2	1.7	NA
LIVER : BODY WEIGHT (%)	Normal	7.2	4.5	3.9	2.7
	Restricted	5.4	3.7	3.2	2
KIDNEY : BODY WEIGHT (%)	Normal	0.6	0.4	0.4	0.3
	Restricted	0.6	0.4	0.4	0.3
HEART : BODY WEIGHT (%)	Normal	0.9	0.7	0.7	0.7
	Restricted	0.9	0.8	0.8	0.7
LUNG : BODY WEIGHT (%)	Normal	0.03	0.03	0.03	0.03
	Restricted	0.03	0.04	0.03	0.02

TABLE 6.2 FETAL BODY : ORGAN WEIGHT RATIOS. Mean fetal organ weights, shown in Table 6.1, were expressed as a percentage of mean total fetal weight at 95, 120, 130 and 140 days gestation, for both normal and growth restricted fetuses. NA = data not obtained. Values for n are as indicated in Table 6.1.

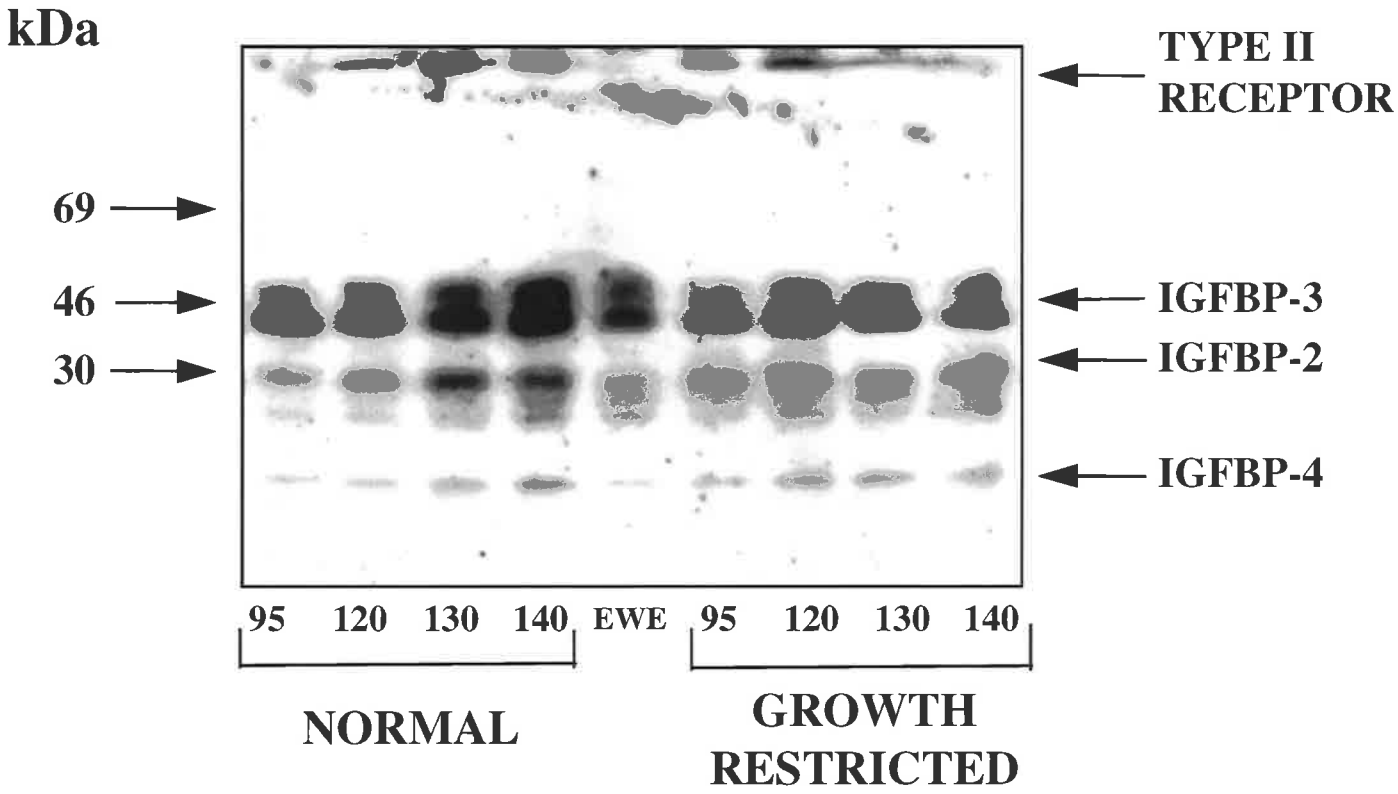


FIGURE 6.2 IGFBPs IN NORMAL AND GROWTH RESTRICTED FETUSES. Representative plasma samples from normal and growth restricted fetuses, at 95, 120, 130 and 140 days gestation were subjected to Western ligand blotting as described in 6.2.2. Plasma from a normal adult ewe plasma pool was electrophoresed for comparison. IGFBP bands representing IGFBP-3, IGFBP-2, IGFBP-4, and possibly the circualting type II IGF receptor, are indicated. The position of molecular weight markers are also indicated.

samples comparing IGFBPs in normal and growth restricted fetuses from 95 to 140 days gestation. Circulating IGFBPs were quantitated (5.2.3) and compared with age matched controls (Figure 6.3). Placental restriction of fetal growth altered circulating IGFBP-2 ($p = 0.0053$), with elevation of circulating IGFBP-2 levels at 95 and 120 days gestation ($p = 0.01$ and $p = 0.02$, respectively) in growth restricted, compared to normal fetuses. Circulating IGFBP-3 was similar in growth restricted fetuses and controls, although levels tended to be lower in growth restricted fetuses at 140 days gestation. Circulating IGFBP-4 was increased by growth restriction ($p = 0.007$), with elevated IGFBP-4 levels in growth restricted compared to normal fetuses, at 120 and 130 days gestation ($p = 0.02$ and $p = 0.0001$).

6.2.3 NORTHERN ANALYSIS OF IGFBP-2 AND IGFBP-4 mRNA

Tissue IGFBP-2 and IGFBP-4 mRNA levels were measured in a limited number of growth restricted fetuses at 95, 120 and 130 days gestation in the liver, kidney, lung and heart. Results for IGFBP mRNA in the fetal liver, kidney and lung are shown in Figures 6.4, 6.5 and 6.6, respectively. Northern blots from growth restricted and control fetuses at the same gestational ages are shown. IGFBP-2 and IGFBP-4 mRNAs were not detectable in the heart from normal or growth restricted fetuses at the gestational ages examined (data not shown). IGFBP-2 mRNA is present at highest levels in the kidney > liver >> heart = lung at the gestational ages studied, in both growth retarded and normal fetuses. IGFBP-4 mRNA was highest in the lung ≥ liver > kidney >> heart in both the growth restricted and control fetuses at 95 to 130 days gestation. Thus, alterations in the tissue specificity or relative mRNA abundance were not induced by placental restriction of fetal growth for either IGFBP-2 or IGFBP-4 mRNA. IGFBP mRNA levels for IGFBP-2 and IGFBP-4 in the liver and kidney and IGFBP-4 in the lung were quantitated by densitometry, normalised for RNA loading against 18S rRNA binding, and standardised against the normal ewe liver control electrophoresed on each gel (Figure 6.7). There were no major changes in IGFBP-2 or IGFBP-4 mRNA abundance in the liver, kidney or lung in growth restricted compared to control fetuses.

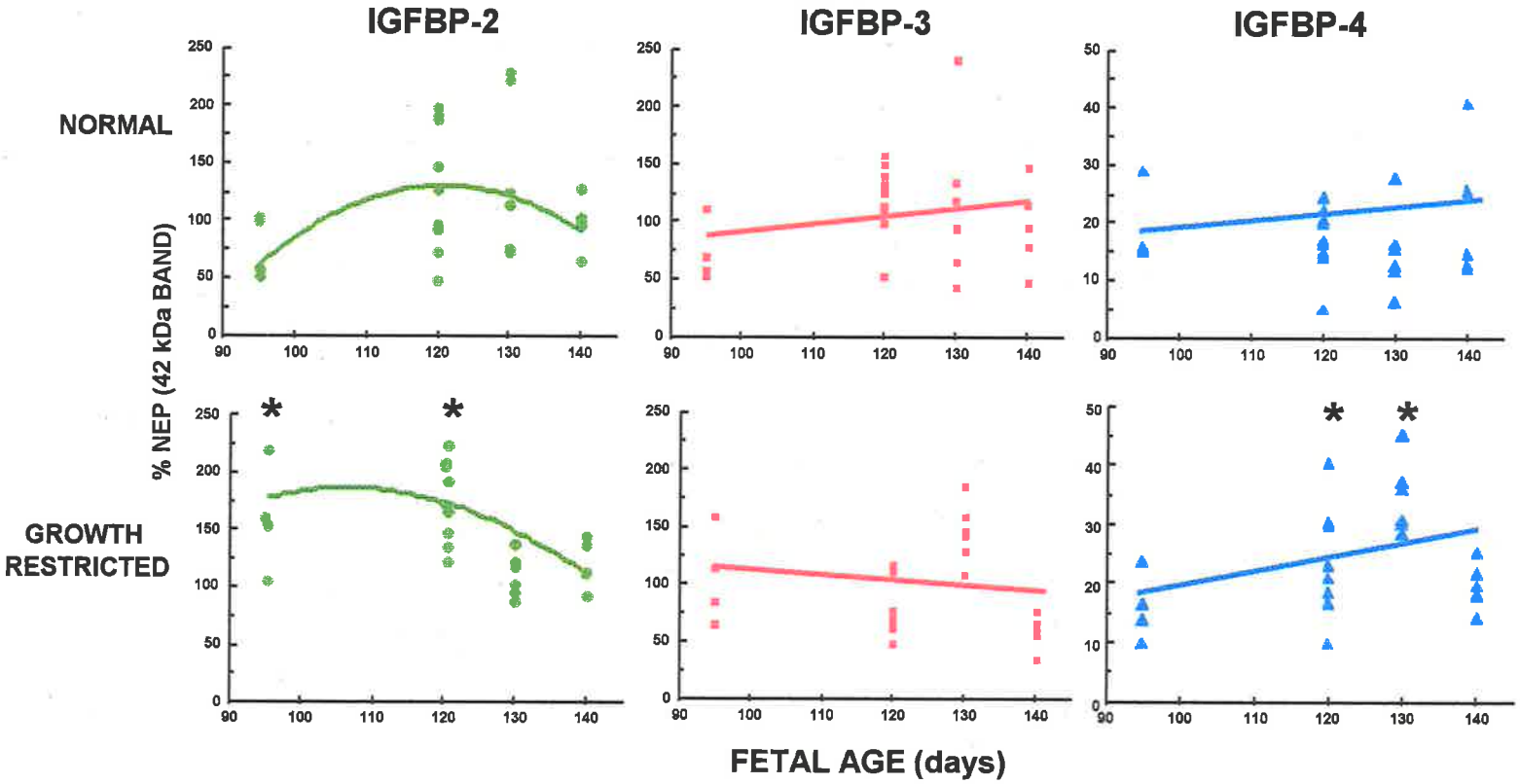


FIGURE 6.3 QUANTITATION OF CIRCULATING IGFBP LEVELS BY WESTERN LIGAND BLOT. Plasma from growth restricted fetuses at 95 (n = 4), 120 (n = 9), 130 (n = 6) and 140 days gestation (n = 6) and normal fetuses (Chapter 5), were subjected to Western ligand blotting and IGFBP-2, IGFBP-3 and IGFBP-4 quantitated, as described in 6.2.2. Band intensities were determined by laser densitometry and values expressed as a percentage of the 42 kDa band of IGFBP-3 from the normal ewe control (NEP). Data were subjected to ANOVA, with specific contrasts between normal and growth restricted values by least squares means. * = significantly different to age matched control values. p (probability) values are indicated in the text. Lines of best fit are indicated (Cricket Software). Line equations and r^2 values are not shown.

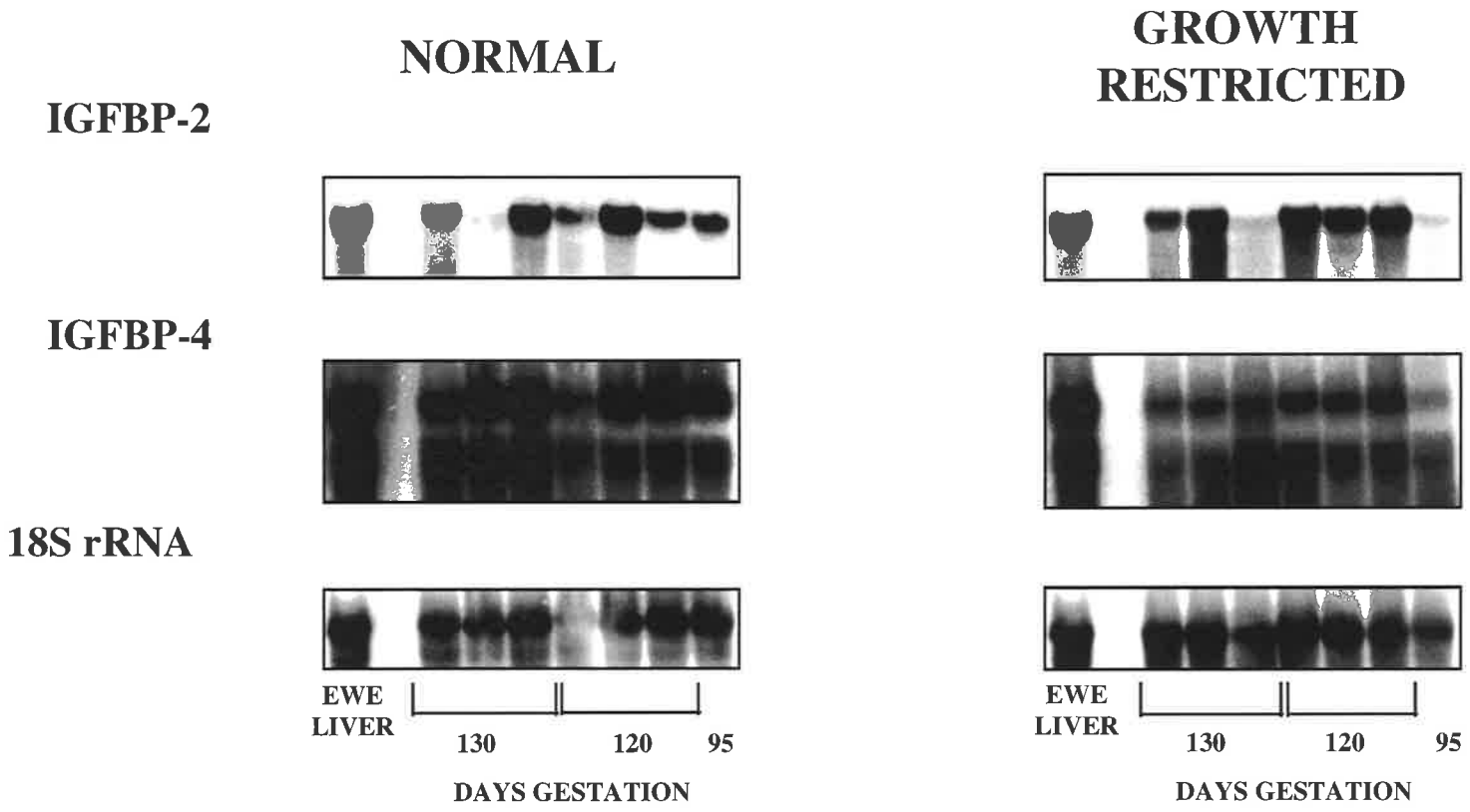


FIGURE 6.4 IGFBP-2 AND -4 mRNA IN THE LIVER. Liver RNA from normal and growth restricted fetuses was extracted and subjected to Northern analysis as described in 6.2.2. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA, stripping the filters in between each probing in boiling water. RNA was analysed from fetuses at 95 (n=1), 120 (n=3) and 130 (n=3) days gestation. RNA from a normal adult ewe liver was electrophoresed as a control.

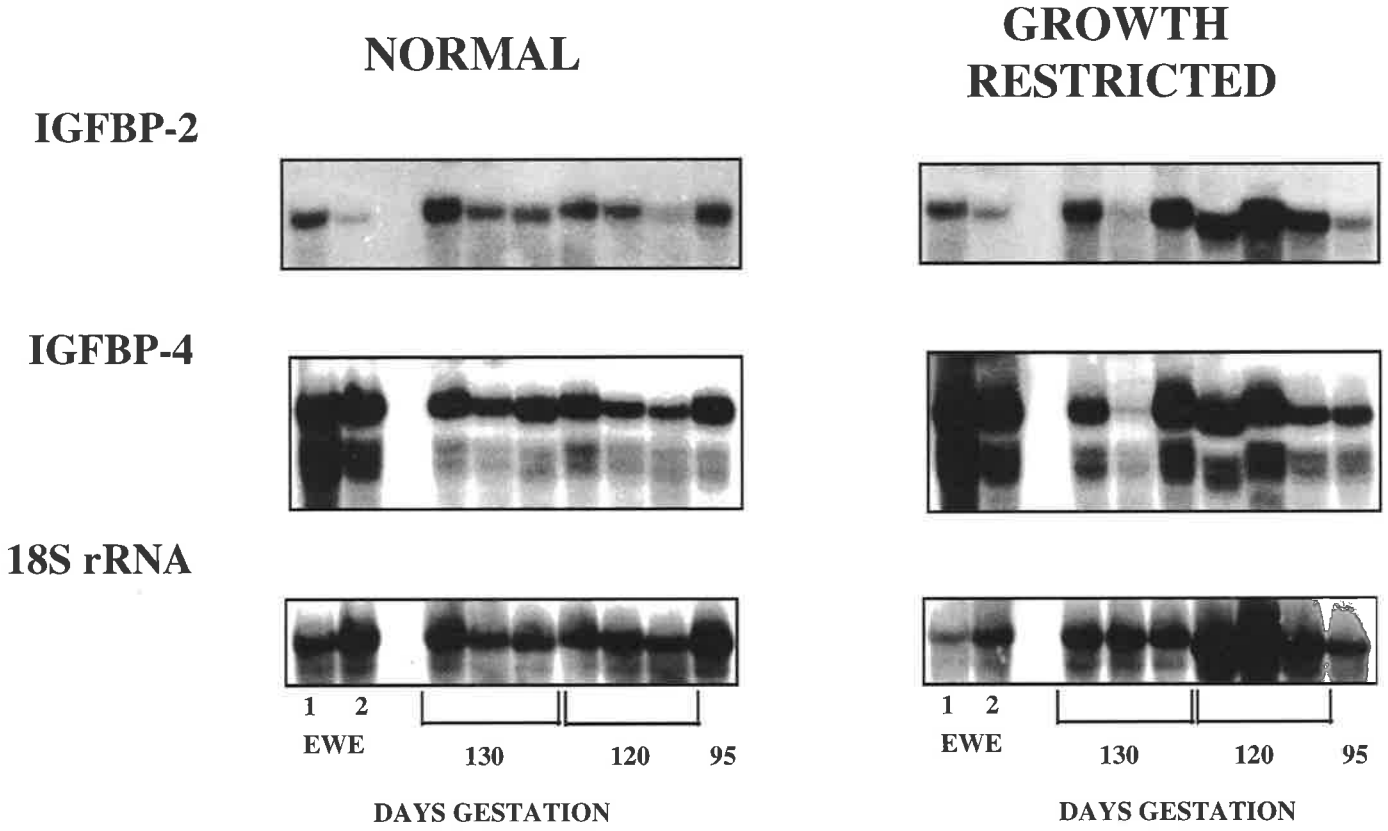


FIGURE 6.5 IGFBP-2 AND -4 mRNA IN THE KIDNEY. Kidney RNA from normal and growth restricted fetuses was extracted and subjected to Northern analysis as described in 6.2.2. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA, stripping the filters in between each probing in boiling water. RNA was analysed from fetuses at 95 (n=1), 120 (n= 3) and 130 (n=3) days gestation. RNA from a normal adult ewe liver (1) and normal adult ewe kidney (2) were electrophoresed as controls.

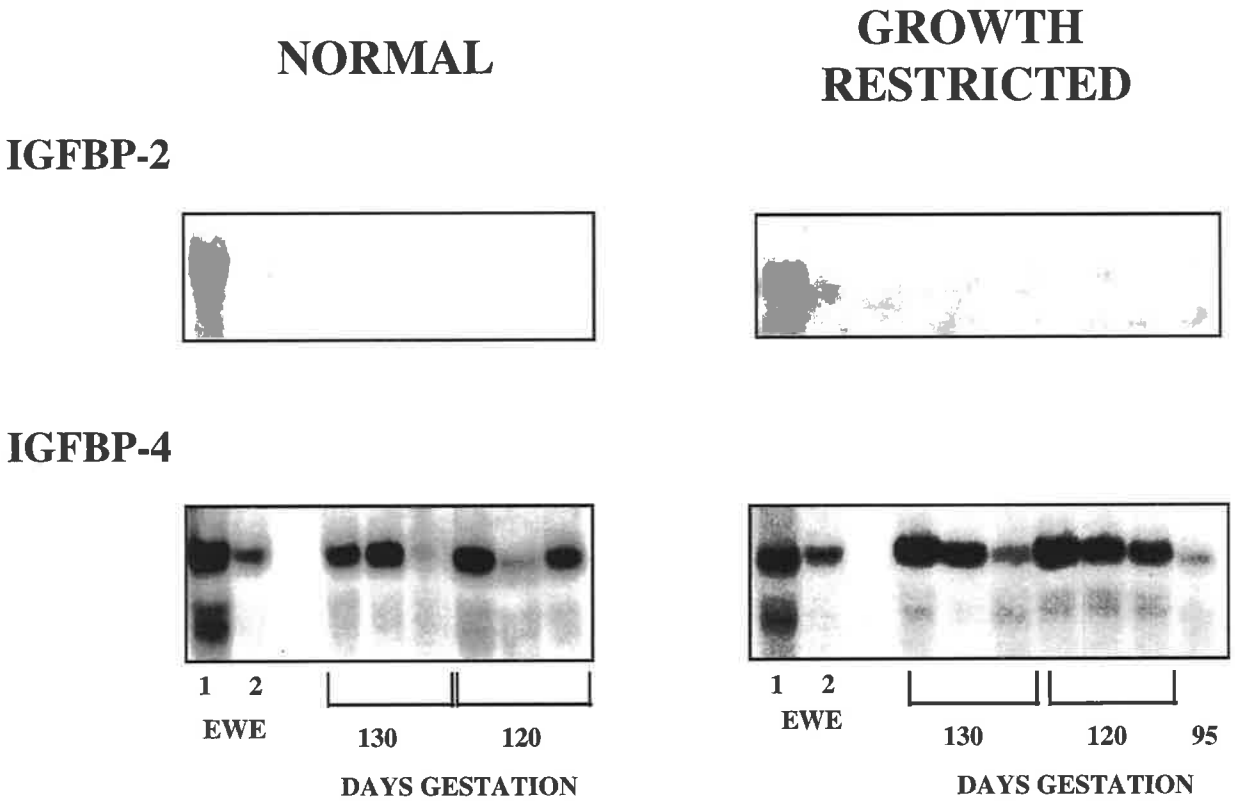


FIGURE 6.6 IGFBP-2 AND -4 mRNA IN THE LUNG. Lung RNA from normal and growth restricted fetuses was extracted and subjected to Northern analysis as described in 6.2.2. Filters were sequentially probed for IGFBP-2, IGFBP-4 and 18S rRNA (data not shown), stripping the filters in between each hybridisation with boiling water. RNA was analysed from fetuses at 95 (n=1), 120 (n=3) and 130 (n=3) days gestation. RNA from a normal adult ewe liver (1) and normal adult ewe lung (2) were electrophoresed as controls.

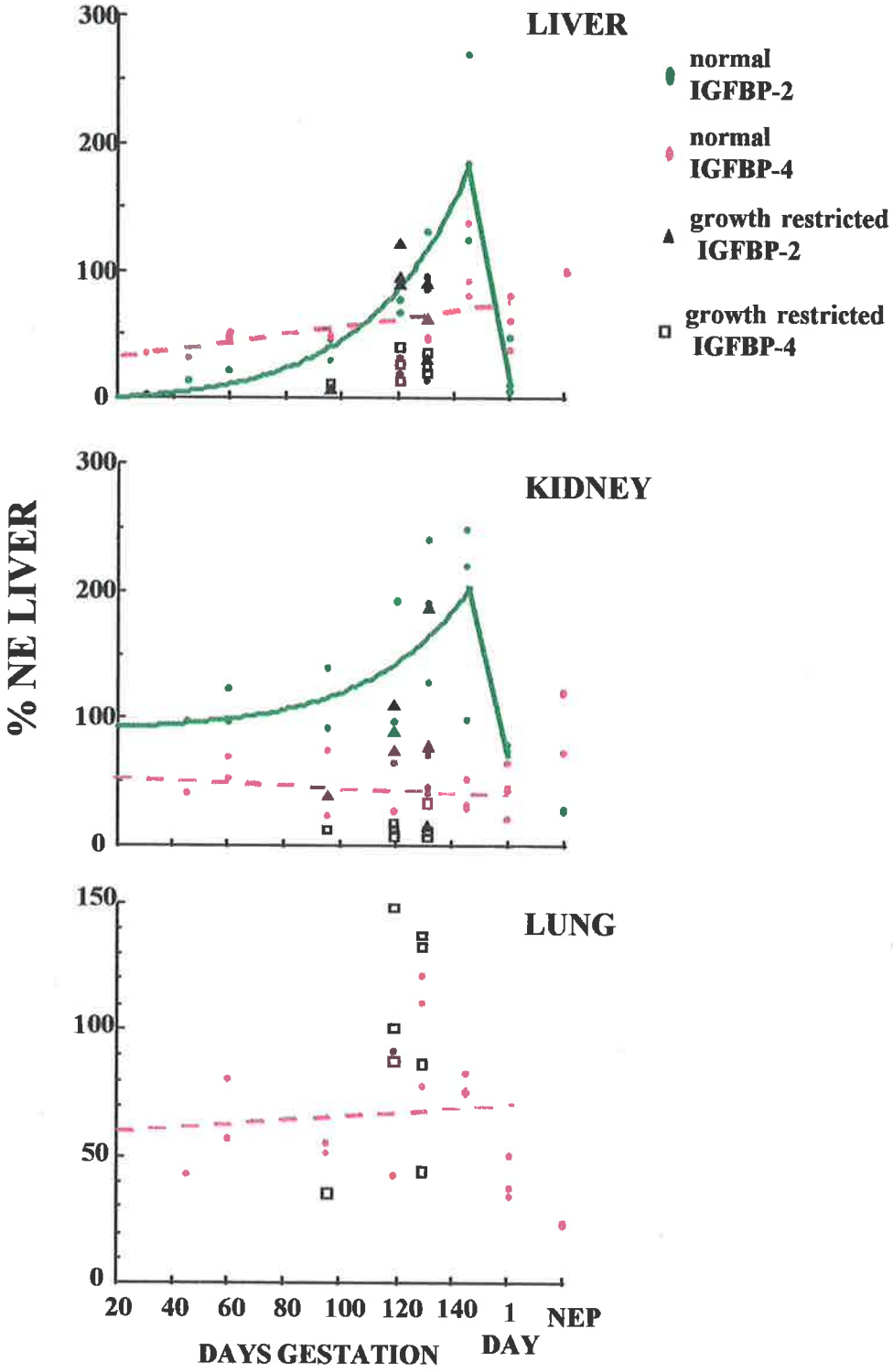


FIGURE 6.7 COMPARISON OF TISSUE mRNA LEVELS OF IGFBP-2 AND IGFBP-4 IN NORMAL AND GROWTH RESTRICTED FETUSES. Autoradiographs from Northern blots for IGFBP-2, IGFBP-4 and 18S rRNA were scanned by laser densitometry, values expressed as a percentage of 18S rRNA then normalised against the adult ewe liver control on each gel. Data was analysed by ANOVA, with specific contrasts by least squares means. Values and regression lines for control animals from Chapter 5 are shown. There was no significant difference in IGFBP-2 or IGFBP-4 mRNA in any tissue of growth restricted compared to normal fetuses.

6.2.4 CORRELATION ANALYSIS

The correlations between levels of circulating IGFBPs, fetal weight and fetal organ weights in growth restricted fetuses from 95 to 140 days gestation, are indicated in Table 6.3. In the normal fetus, circulating IGFBP-3 and IGFBP-4 and IGFBP-2 are positively correlated with fetal weight (Chapter 5). In the growth restricted fetus, fetal weight was negatively correlated with circulating IGFBP-2. In addition, circulating IGFBP-2 also correlated negatively with weight of the fetal kidney and lung, while correlations of IGFBP-2 with weight of the fetal heart and liver approached significance ($r = -0.39$, $p = 0.06$ and $r = -0.40$, $p = 0.058$, respectively). This suggests an inhibitory effect of circulating IGFBP-2 on fetal growth. The higher molecular weight or glycosylated form of IGFBP-3, but not total IGFBP-3 positively correlated with weight of the growth restricted fetus ($r = 0.5$, $p = 0.03$), while placental weight, cotyledon number and circulating IGFBP-4 were positively associated with total circulating IGFBP-3 in the growth restricted fetus. The correlation between circulating IGFBP-3 and fetal liver weight approached significance ($r = 0.54$, $p = 0.086$). Circulating IGFBP-4 was positively correlated with circulating IGFBP-3 and fetal liver, kidney and lung weights (Table 6.3).

Circulating or tissue mRNA for IGFBP-2 showed no association with fetal blood substrate levels, while circulating IGFBP-4 was positively correlated with pH ($r = 0.43$, $p = 0.04$) and circulating IGFBP-3 was positively correlated with pO_2 ($r = 0.45$, $p = 0.03$) and oxygen saturation ($r = 0.46$, $p = 0.03$). Circulating concentrations of glucose, insulin, IGF-I and IGF-II were available from a subset of the animals used in this study, but showed no association with fetal body or organ weights or circulating or tissue mRNA levels of IGFBPs. However, insulin was negatively correlated with placental weight ($r = -0.64$, $p = 0.03$), circulating IGFBP-3 ($r = -0.74$, $p = 0.006$) and lung IGFBP-4 mRNA ($r = 0.98$, $p = 0.003$) at 120 and 130 days gestation. Circulating IGFBP-2 and IGFBP-4 were not correlated with IGFBP-2 or IGFBP-4 mRNA in any tissue examined. Tissue mRNA abundance for IGFBP-2 or IGFBP-4 was not associated with fetal weight or the weight of fetal organs, except in the kidney, where kidney weight was positively correlated with kidney IGFBP-2 mRNA abundance.

		FETAL WEIGHT	PLAC. WEIGHT	COT. #	LIVER WEIGHT	KIDNEY WEIGHT	HEART WEIGHT	LUNG WEIGHT	CIRC. IGFBP-2	CIRC. IGFBP-3	CIRC. IGFBP-4
FETAL WEIGHT	r p	-	*ns*	0.7 0.001	0.7 0.0002	0.84 0.001	0.95 0.0001	0.87 0.0001	-0.43 0.03	*ns*	*ns*
PLAC. WEIGHT	r p	*ns*	-	*ns*	0.55 0.005	*ns*	*ns*	*ns*	*ns*	0.675 0.0003	*ns*
COT. #	r p	0.70 0.001	*ns*	-	0.63 0.005	0.69 0.002	0.77 0.0002	0.59 0.01	-0.48 0.05	0.61 0.007	0.61 0.007
LIVER WEIGHT	r p	0.7 0.0002	0.55 0.005	0.63 0.005	-	0.84 0.0001	0.75 0.0001	0.84 0.0001	*ns**	*ns**	0.52 0.01
KIDNEY WEIGHT	r p	0.84 0.001	*ns*	0.69 0.002	0.84 0.0001	-	0.87 0.0001	0.83 0.0001	-0.54 0.006	*ns*	0.42 0.04
HEART WEIGHT	r p	0.95 0.0001	*ns*	0.77 0.0002	0.75 0.0001	0.87 0.0001	-	0.84 0.0001	*ns*	*ns*	*ns*
LUNG WEIGHT	r p	0.87 0.0001	*ns*	0.59 0.01	0.84 0.0001	0.83 0.0001	0.84 0.0001	-	-0.43 0.04	*ns*	0.42 0.04
CIRC. IGFBP-2	r p	-0.43 0.033	*ns*	-0.48 0.05	*ns**	-0.54 0.006	*ns*	-0.43 0.04	-	*ns*	*ns*
CIRC. IGFBP-3	r p	*ns*	0.68 0.0003	0.61 0.007	*ns**	*ns*	*ns*	*ns*	*ns*	-	0.58 0.002
CIRC. IGFBP-4	r p	*ns*	*ns*	0.61 0.007	0.52 0.01	0.42 0.04	*ns*	0.42 0.04	*ns*	0.58 0.002	-
LIVER -2 mRNA	r p	*ns*	*ns*	*ns*	*ns*	ns	*ns*	*ns*	*ns*	-0.84 0.02	*ns*
KIDNEY -2 mRNA	r p	*ns*	*ns*	*ns*	*ns*	0.77 0.04	*ns*	*ns*	*ns*	*ns*	*ns*
LIVER -4 mRNA	r p	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
KIDNEY -4 mRNA	r p	*ns*	0.89 0.008	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LUNG -4 mRNA	r p	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	-0.77 0.04	*ns*

TABLE 6.3 CORRELATION ANALYSIS. Circulating and tissue mRNA levels for IGFBPs in the growth restricted fetus were correlated with fetal body and organ weights. Brain weight was not significantly associated with any parameter. Correlations with circulating substrate levels are described in the text. * indicates correlation approaches significance and is described in the text. *ns* = not significant.

6.3 DISCUSSION

This study has shown that intra-uterine growth retardation in the sheep, induced by restriction of placental implantation and substrate supply, is characterised by changes in circulating levels of IGFBPs in the fetus, between mid to late gestation. Circulating IGFBP-2 levels are increased by mid gestation following restriction of placental growth, before significant restriction of fetal growth is apparent. Together with the negative associations of circulating IGFBP-2 with fetal weight and weight of fetal organs, this suggests that circulating IGFBP-2 may inhibit fetal growth and be involved in the initial onset of fetal growth restriction. However, kidney IGFBP-2 mRNA abundance correlated positively with fetal kidney weight in the growth restricted fetus, perhaps suggesting that although endocrine IGFBP-2 may act as a growth inhibitor, paracrine or autocrine IGFBP-2 may have local growth stimulating effects.

The ontogeny of circulating IGFBP-3 was not altered by restriction of placental growth. However, measurements of circulating immunoreactive IGFBP-3 in the growth restricted fetus, using the specific oIGFBP-3 RIA, as described in Chapter 5, has shown that IGFBP-3 levels are reduced in a larger cohort of growth restricted fetuses at 95 and 120 days gestation compared to controls (J. A. Owens, Department of Obstetrics and Gynaecology, University of Adelaide, pers. comm.). Since IGFBP-3 is also correlated with fetal and placental weights, this is consistent with a positive role for IGFBP-3 in regulating growth *in utero*.

Circulating IGFBP-4 was increased at 120 and 130 days gestation in the growth restricted fetuses, in comparison to age matched controls, although levels of circulating IGFBP-4 in growth restricted fetuses were positively correlated with several fetal organ weights. This may suggest that circulating IGFBP-4 is associated with promoting organ growth. The elevated circulating IGFBP-4 levels in the growth restricted fetus at 120 and 130 days gestation, may enhance or direct the delivery of IGFs to tissues of the restricted fetus, stimulating the growth of these organs. Thus, the high levels of circulating IGFBP-4 may not be inhibitory to IGF actions and it may be the reduced levels of IGF and elevated levels of other IGFBPs observed in these fetuses that are responsible for the retardation of

growth in comparison to controls.

The tissue distribution, or relative mRNA levels for either IGFBP-2 or IGFBP-4 mRNA were unchanged in a subset of growth restricted fetuses in comparison to controls. IGFBP-2 mRNA was not present in the heart or lung, but was readily detectable in the liver or kidney, of growth restricted fetuses between 95 and 130 days gestation, as is seen in the normal fetus. IGFBP-4 mRNA was not present in the heart, but is detectable in the liver, kidney and lung of growth restricted fetuses between 95 and 130 days gestation, again, the same as is observed in the normal fetus. Levels of IGFBP-2 or IGFBP-4 mRNA in the liver and kidney or IGFBP-4 mRNA in the lung was unchanged in growth restricted fetuses compared to controls. Thus, the changes observed in circulating IGFBP-2 and IGFBP-4 in the growth restricted fetuses are not reflected by alterations in mRNA abundance in the major fetal organs. Since restriction of growth did not dramatically alter IGFBP mRNA levels, with the low number of animals used in this study, small changes in mRNA abundance may not be readily detected. Additionally, to assess more subtle changes in mRNA levels, a more sensitive and quantitative assay, such as the RNase protection assay, would be more appropriate. Analysis of IGFBP-2 and IGFBP-4 mRNAs in the tissues of the growth retarded fetuses should be pursued further with increased numbers of observations and a more sensitive method of analysis.

Previous studies of fetal growth retardation in the rat and sheep suggest a role for IGF-I and IGFBP-1 in the initial response to substrate restriction, while the responses of IGF-II and IGFBP-2 may be more complex. In IUGR in the rodent, induced by maternal fasting, uterine artery ligation, dexamethasone administration or maternal hypoxia, circulating levels and hepatic mRNAs for IGF-I decrease and IGFBP-1 increase, but the responses of IGF-II or IGFBP-2 are variable with reported increases in IGF-II or liver IGFBP-2 mRNA (Jones *et al.*, 1987; Price *et al.*, 1992; Tapanainen *et al.*, 1994) decreases in liver IGF-II mRNA (Straus *et al.*, 1991) or no change in either of these factors (Unterman *et al.*, 1993a and 1993 b) (1.3.4). Circulating IGFBP-2 is increased in the neonatal rat subjected to maternal dietary restriction during lactation (Donovan *et al.*, 1991) and the growth restricted fetal rat induced by maternal hypoxia (Tapanainen *et al.*, 1994).

Conversely, circulating IGFBP-2 is decreased in the acute hypoxic sheep fetus (McLellan *et al.*, 1992). In contrast to these variable responses seen for IGFBP-2 in most other models of growth restriction, growth restriction induced by placental restriction of substrate supply increased circulating levels of IGFBP-2, but not IGFBP-1.

IGFBP-1 has been shown by several studies to be induced in response to hypoglycaemia, hypoxia and high levels of catecholamines and glucocorticoids in the rat or sheep (McLellan *et al.*, 1992; Rechler, 1993; Hooper *et al.*, 1994) and thus the rapid increase in IGFBP-1 in other acute models of growth restriction may relate to rapid changes in fetal blood glucose, insulin or oxygen content. Although IGFBP-1 was not specifically measured in this study (since normal levels of this IGFBP are not high enough to reliably quantitate by Western ligand blot and densitometry), there was no induction of any IGFBP band in the expected molecular weight region for IGFBP-1 (detected by Western ligand blotting) in growth restricted fetuses compared to controls. IGFBP-1 has previously been visualised by Western ligand blotting in ovine samples from six week old lambs subjected to extended anaesthesia and surgery (data not shown) and thus the lack of observation of a major induction of IGFBP-1 is not due to the technical inability to resolve IGFBP-1 from IGFBP-2. However, subtle alterations in IGFBP-1 levels or phosphorylation state, not detectable by Western ligand blotting, cannot be excluded.

In the perturbations of the intra-uterine environment described previously, growth restriction is induced over a short period of time, relative to gestational length, in the late gestation rodent. The responses of the IGF / IGFBP axes to chronic growth restriction and reduced substrate supply over a longer period of time in a larger mammalian species throughout mid to late gestation, as in the model of IUGR described herein, may differ. Therefore, the contrasting results for circulating IGFBP-1 and IGFBP-2 between most previous studies of fetal growth restriction and those described in this study may relate to the different models of growth restriction, involving chronic versus acute adaptive responses to metabolic changes. Although IGFBP-1 is primarily considered to be the insulin responsive IGFBP, involved in adaptation to metabolic changes, IGFBP-2 is also induced by low insulin levels (Rechler, 1993). Ooi *et al.* (1992) have proposed that IGFBP-1 responds

to acute changes, while IGFBP-2 is involved in chronic responses to alterations in insulin and glucose levels (1.2.5.1). IGFBP-2 is also induced in other physiological situations involving chronic exposure to low insulin and glucose levels, such as tumour associated hypoglycaemia in humans (Zapf *et al.*, 1990). Although circulating glucose and insulin levels were not measured in all samples in this study, fetal growth restriction induced by placental restriction of implantation in the sheep has previously been shown to be characterised by low circulating fetal insulin and glucose levels. Thus, results from this study indicate elevated circulating IGFBP-2 levels associated with extended hypoinsulinaemia and hypoglycaemia, *in utero,* and support the proposed role of IGFBP-2 as a chronic counter-regulator of insulin and glucose levels. However, circulating IGFBP-3, but not IGFBP-2, was associated with circulating insulin levels in the growth restricted fetus at 120 and 130 days gestation. Measurements of glucose and insulin levels in all these animals should be completed to confirm these associations.

The elevated levels of IGFBP-2, in the mid gestation growth restricted fetus, are not maintained throughout later gestation, where they declined to control values. In the normal fetus, circulating IGFBP-2 levels decline in late gestation and it was hypothesised that this decline may be mediated by the pre-partum rise in circulating fetal cortisol or a decline in circulating IGF-II (Chapter 5). Previous reports suggest that circulating cortisol levels are elevated in this model of fetal growth restriction (Harding *et al.*, 1985; Robinson *et al.*, 1994). Thus, the decline in circulating IGFBP-2 between 120 and 130 days gestation in the growth restricted fetus may be analogous to the pre-term decline in circulating IGFBP-2 seen in the normal fetus, and result from increased levels of circulating fetal cortisol. In contrast, the initial elevation in circulating levels of IGFBP-2 in the growth restricted fetus may be a result of chronic hypoglycaemia and hypoinsulinaemia, *in utero*. Measurements of glucose, insulin and cortisol in the restricted fetus are required to confirm this hypothesis.

Changes in circulating IGFBP-4 in the growth restricted fetus have recently been reported in the growth retarded fetal rat, induced by maternal hypoxia (Tapanainen *et al.*, 1994). Similar to the results described in this chapter, circulating IGFBP-4 levels are increased in the growth retarded fetus induced by maternal hypoxia (Tapanainen *et al.*,

1994). However, in the current study, circulating IGFBP-4 is only elevated in growth restricted fetus at 120 and 130 days gestation, in comparison to controls. The mechanisms of this response are unclear, although there appears to be no obvious elevation in IGFBP-4 mRNA levels in any of the major fetal tissues. Several studies have described proteases for IGFBP-4, whose action is stimulated by IGFs in a receptor and cell-independent manner (Fowlkes and Freemark, 1992a; Neely and Rosenfeld, 1992; Myers *et al.*, 1993; Kanzaki *et al.*, 1994) (1.2.5.2). Although there is no evidence for the presence of these proteases in the fetal circulation, a decrease in circulating IGFs in the growth restricted fetus, may result in decreased proteolytic activity for IGFBP-4, leading to increases in circulating IGFBP-4 levels. Alternatively, there may be changes in IGFBP-4 mRNA, not detected in this study, induced by hormones such as progesterone, for which there is a proposed regulatory element in the rat IGFBP-4 promoter (Gao *et al.*, 1993). Such a stimulus may increase IGFBP-4 specifically at 120 and 130 days gestation.

One possibility not addressed in this study is that the changes in IGFBPs in the fetal circulation may be due to alterations in placental production of IGFBPs. The correlation between placental weight and circulating IGFBP-3 levels may suggest that the placenta is a primary source of circulating IGFBP-3 or that IGFBP-3 may regulate placental growth.

In conclusion, restriction of fetal growth is associated with changes in the ontogeny and circulating levels of some IGFBPs, with increases in circulating IGFBP-2 in mid gestation, a premature decline in IGFBP-2 in late gestation, and increases in circulating IGFBP-4 specifically at 120 and 130 days gestation. The changes in circulating IGFBP-2 were inversely related to fetal body and organ weights, suggesting inhibition of fetal growth by IGFBP-2. Since growth retardation resulting from placental restriction of substrate supply is asymmetric (1.3.4) and the IGFBPs have been suggested to play a role in specific tissue distribution and localisation of IGFs (1.2.3.2), the specific changes observed in circulating IGFBPs in the growth retarded fetus may relate to the asymmetry of growth restriction, through a redistribution or altered delivery of IGF to specific target tissues. Thus, altered delivery and distribution of IGFs, in conjunction with a reduction in the endocrine growth potential of the fetus, through increased levels of some IGFBPs (growth

inhibitors), particularly IGFBP-2, and reduced abundance of IGFs (growth promoters), may be partly responsible for the observed perturbation of fetal growth.

CHAPTER 7. CHARACTERISATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs) IN THE TAMMAR WALLABY, *MACROPUS EUGENII*

7.1 INTRODUCTION

The insulin-like growth factors and their binding proteins (IGFs and IGFBPs) have been suggested to be important regulators of fetal growth and development in eutherian mammals (1.3.2, 1.3.3, Chapters 5 and 6). Although the IGFs and IGFBPs are strongly conserved between species both in terms of primary sequence and regulation (Rechler, 1993), there are major differences in the developmental patterns of IGFs and IGFBPs between species such as the human or sheep compared with rodents. For example, in the rat, IGF-II and IGFBP-2 are high in the fetus and neonate and decline at approximately day 20 post-partum to negligible levels in the adult (Moses *et al.*, 1980; Donovan *et al.*, 1989). In contrast, in the sheep, the decline in IGF-II and IGFBP-2 occurs at birth and significant concentrations of both molecules persist in the adult circulation (Mesiano *et al.*, 1989; Gluckman and Butler, 1983; Chapter 5). In both species this decline in IGF-II occurs concomitantly with a rise in cortisol or glucocorticoids (Beck *et al.*, 1988; Levinovitz and Norstedt, 1989; Li *et al.*, 1993), suggesting common underlying mechanisms controlling the developmental profile of the IGF-IGFBP axes in the sheep and rat, even though initially developmental profiles between these two species appear different. The growth and development of the fetus and young in eutherian mammals are very different to that of marsupials. In eutherian mammals, gestation is usually long and lactation relatively short. However in the marsupial *Macropus eugenii*, the gestational period is short with the fetus being born more immature, after only 28 days gestation. Consequently most of the growth and development of the marsupial young occurs in the pouch during the lactation period, which lasts approximately 260 days. The young begins grazing at around 180 days, but is not completely weaned until approximately 300 days after birth (Tyndale-Biscoe and Janssens, 1988). Many organ systems such as the lungs and kidney (Baudinette *et al.*, 1988; Wilkes and Janssens, 1988) and functional systems, such as locomotor and sensory systems

(Hughes and Hall, 1988) are relatively well developed as an adaptation for pouch life. However, even these systems undergo further development and maturation in the pouch. Comparison of the important modulators of growth, such as the IGFs and IGFBPs, in marsupial and eutherian mammals may identify those aspects essential for mammalian growth and development.

Since manipulation of the eutherian mammalian fetus *in utero* requires invasive surgery, the accessibility of the developing marsupial young also has technical advantages. Biological fluids can be readily sampled, exogenous factors directly injected or infused, nutritional input can be controlled by manipulating lactation and the young can be sacrificed at desired developmental time points, without surgical or hormonal intervention to the mother. The advantages of marsupials as developmental models have been recognised previously (Tyndale-Biscoe and Janssens, 1988) and used to investigate the development of systems such as the spinal cord (Harrison and Porter, 1992) and thyroid function (Janssens *et al.*, 1990).

This study has characterised the IGFBPs present in the developing marsupial pouch young and lactating mother in order to further elucidate the roles of the insulin-like growth factors IGFs and IGFBPs in growth and development. Previous studies in our laboratory have established the presence of IGFBPs by Western ligand blotting in the marsupial *Sminthopsis crassicaudata* (Upton *et al.*, 1993b). However, this investigation has utilised the tammar wallaby, *Macropus eugenii,* a species that has been extensively investigated for other aspects of reproductive biology.

7.2 MATERIALS AND METHODS

7.2.1 MATERIALS

IGFBP-3 and -4 antibodies were a kind gift from Dr. P. E. Walton and Mrs P. A. Grant, CRC for Tissue Growth and Repair, Adelaide, South Australia. Bovine IGFBP-2 antibodies were developed in our laboratory by Mr. L. Szabo. Gamma globulin, carbonic anhydrase, chymotrypsinogen and lysozyme were obtained from Sigma Chemical Co., St Louis, MO, USA. All other reagents are as described in section 2.1.

7.2.2 BLOOD AND TISSUE SAMPLING

All biological samples were obtained from an established *M. eugenii* colony at the School of Biological Sciences, Flinders University, Bedford Park, South Australia. Samples were obtained from a non-reproductive female (n = 1), adult males (n = 3), non-lactating adult females (n = 3), pouch young and mothers; 10 days, n = 2; 50 days, n = 2; 100 days, n = 2; 185 days, n = 1; 220 to 230 days, n = 2. Blood from adults and pouch young older than 100 days were obtained via a tail vein and collected into heparinised microcapillaries or tubes. Plasma was prepared by centrifugation at 2,000 x g for 10 minutes at room temperature. Blood from younger wallabies was obtained by heart puncture after sacrifice by intra-peritoneal phenobarbitone overdose, collected into heparinised microcapillaries and the plasma prepared as described above. Tissue samples from these animals were collected immediately into liquid nitrogen. The age of pouch young was estimated from head length measurement; the data base having been established from many animals over a five year period (R. V. Baudinette, School of Biological Science, Flinders University, Adelaide, South Australia, personal communication). Additionally, samples were obtained from a 50 day pouch young (#181), subjected to phenobarbitone overdose and lung perfusion prior to removal of tissues and collection of blood by severing the jugular vein. Sheep plasma was obtained from non-pregnant adult ewes by jugular venipuncture (n = 3), as described in 5.2.2.

7.2.3 WESTERN LIGAND BLOTTING

Plasma (20 µl) was diluted 1/10 in PBS, and an aliquot (20 or 40 µl; 2 or 4 µl plasma equivalents) was subjected to Western Ligand blotting with ^{125}I-IGF-II and filters autoradiographed for four weeks at -80^{o} C (2.2.3.1). Molecular weights of IGFBP bands were calculated from a standard curve of migration distances of molecular weight markers (data not shown). Filters were then reprobed with antibodies to ovine IGFBP-3, ovine IGFBP-4 and bovine IGFBP-2 all at 1/500 dilution of serum (2.2.3.2).

7.2.4 NEUTRAL GEL FILTRATION

Plasma (50 µl) was pooled from animals of the same age and incubated overnight with approximately 60,000 cpm ^{125}I-IGF-II or ^{125}I-IGF-I in a total volume of 60 µl at 4° C. Equilibrated plasma was then subjected to neutral gel filtration on a 10 x 300 mm Superose 6 column (Pharmacia, Uppsala, Sweden) in 50 mM sodium phosphate, pH 7.2, 0.15 M NaCl, 0.02 % sodium azide. Chromatography was performed at 0.3 ml/min and fractions collected at one minute intervals. The column was calibrated with gamma globulin (160 kDa), BSA (69 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen (24 kDa), lysozyme (14.3 kDa) and ^{125}I-IGF (7.5 kDa). Fractions were counted (LKB Wallac 1261 Multigamma Counter, LKB, Turku, Finland). Fractions from lactating mothers (50 days, n = 2 pooled) and a pouch young (220 to 230 day, n = 2 pooled) were dialysed (Spectra/Por membrane molecular weight cut off 8 kDa, Spectrum Medical Industries, Inc, CA, USA) in 10 mM Tris overnight at 4° C and subjected to Western ligand blotting as described above.

7.2.5 NORTHERN ANALYSIS OF TOTAL LIVER RNA

Total RNA was extracted from *M. eugenii* pouch young by the method of Chomczynski and Sacchi (1987), (2.2.2.1) and liver RNA (40 µg) was subjected to Northern analysis (2.2.2.2). Membranes were washed at increasing temperature from 42° C, 55° C to 60° C in 2 X SSC + 0.1 % (w/v) SDS. Filters were sequentially probed with rat IGFBP-1 (Murphy *et al.*, 1990), bovine IGFBP-2 (Upton *et al.*, 1990), rat IGFBP-3 (Albiston and Herington, 1990) and ovine IGFBP-4 (Carr *et al.*, 1994a). Membranes were stripped in between each probing with boiling water for 60 minutes. RNA loading was assessed by reprobing with an 18S rRNA genomic clone (Katz *et al.*, 1983). Autoradiographs were exposed to x-ray film at -80° C for 2 weeks, 10 days, 12 days and overnight, for IGFBP-1, -2, -4 and 18S rRNA, respectively. The size of mRNA transcripts was determined by comparison to ovine IGFBP transcripts and from a standard curve constructed from an RNA ladder (data not shown).

7.3 RESULTS

7.3.1 CHARACTERISATION OF WALLABY IGFBPs

Western ligand blotting of wallaby plasma from male and female adults, a 230 day pouch young and its mother revealed five bands of approximate molecular weights : 42 to 50 kDa (doublet), 30 kDa, 28 kDa and 24 kDa (Figure 7.1). The apparent molecular weights of these IGFBPs suggest that they may represent wallaby IGFBP-3 (42 to 50 kDa), IGFBP-1 or -2 (30 kDa) and IGFBP-4 (28 and 24 kDa). Alternatively, the smaller molecular weight species may be IGFBP-5 or IGFBP-6. A band of approximately 200 kDa was often visible in plasma from the pouch young and lactating mother, which may represent the circulating type II IGF/cation-independent mannose 6-phosphate receptor. Immunoblotting using antibodies to ovine IGFBP-3 and -4 and bovine IGFBP-2 failed to positively identify any wallaby IGFBPs (data not shown).

Neutral gel filtration of plasma from adult males or females following incubation with ^{125}I-IGF-I or ^{125}I-IGF-II indicates binding activity at 70 to 160 kDa and 25 to 60 kDa (Figure 7.2A and 7.2B). This binding activity is specific as shown by competition with unlabelled IGF-II and comparable to that observed for adult ewe plasma (Figure 7.2C). The reduced amount of IGF-I bound to the 70 to 160 kDa region observed in female compared to male adult wallabies also occurs in the sheep (P. A. Grant, CRC for Tissue Growth and Repair, Adelaide, pers. comm.). The small amount of radioactivity eluting in the void volume of greater than 160 kDa appears to be non-specific binding, as judged by its presence in both IGF-I and IGF-II chromatograms and the lack of competition with unlabelled IGF-II (Figure 7.2A and 7.2B). However, there is a high molecular weight species particularly abundant in samples from lactating mothers or pouch young, an example of which is shown in Figure 7.2D. Fractions were collected from the chromatograms shown in Figure 7.2D, dialysed and subjected to Western ligand blotting (Figure 7.3). Results indicate a high molecular weight species in the region >160 kDa, which may be the soluble type II IGF/cation-independent mannose 6-phosphate receptor. The 70 to 160 kDa binding region on neutral gel filtration, electrophoresed under denaturing conditions predominantly as the 42 to 50 kDa doublet, presumably IGFBP-3. All other bands, as identified in Figure

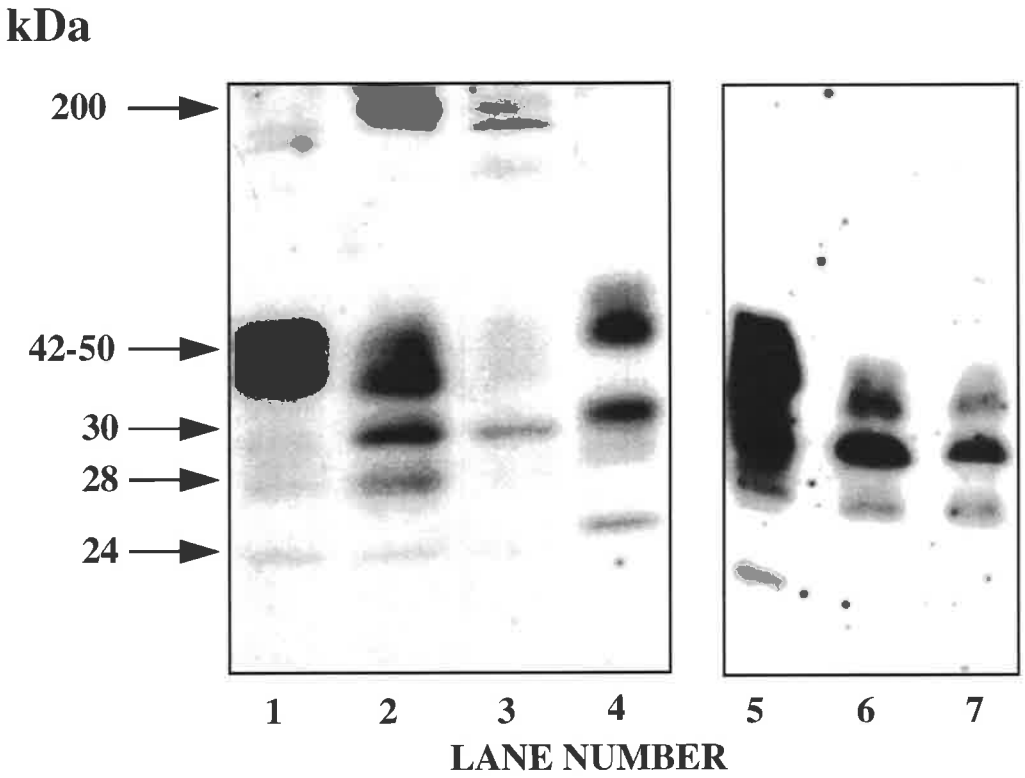


FIGURE 7.1 WESTERN LIGAND BLOT OF WALLABY PLASMA. Plasma samples were subjected to SDS-PAGE and Western ligand blotting as described in 7.2.3. Lane 1 = normal female human plasma (2 µl); Lane 2 = 230 day pouch young (2 µl); Lane 3 = 230 day lactating wallaby mother (2 µl); Lane 4 = normal ewe plasma (2 µl); Lane 5 = normal ewe plasma (2µl); Lane 6 = non-lactating adult female wallaby (4 µl); Lane 7 = adult male wallaby (4 µl). Calculated molecular weights of IGFBP bands are indicated. Autoradiographs were exposed for two weeks (Lanes 1 to 4) and one month (Lanes 5 to 7).

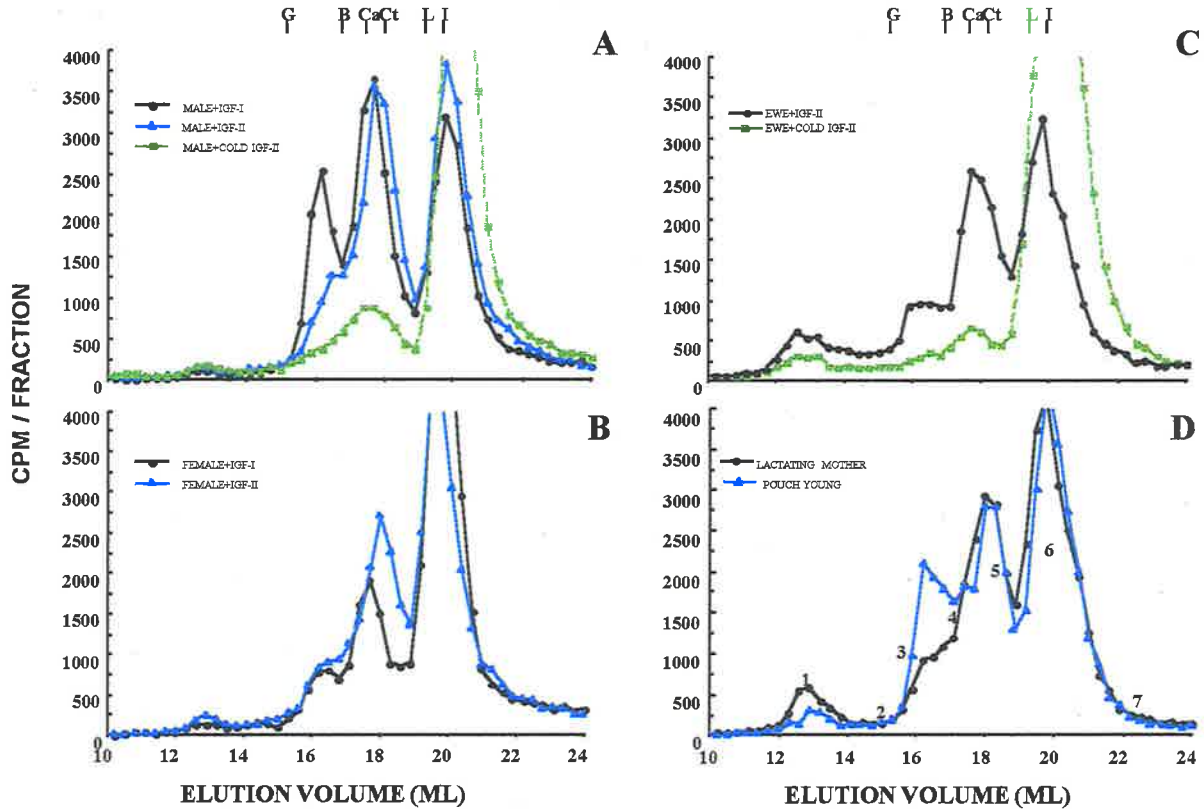


FIGURE 7.2 NEUTRAL GEL FILTRATION OF WALLBY PLASMA. Plasma (50 μl) was incubated with ^{125}I-IGF-I or ^{125}I-IGF-II and subjected to neutral gel filtration (7.2.4). A = adult male wallaby plasma (n = 3, pooled) incubated with ^{125}I-IGF-I (●), ^{125}I-IGF-II alone (▲) or co-incubated with 1 μg of unlabelled IGF-II (■). B = non-lactating female wallaby plasma (n = 3, pooled) incubated with ^{125}I-IGF-I (●) or ^{125}I-IGF-II (▲). C = normal adult ewe plasma (n = 3, pooled) incubated with ^{125}I-IGF-II alone (●) or co-incubated with 1 μl unlabelled IGF-II (■). D = lactating wallaby mothers plasma (50 days, n = 2, pooled) incubated with ^{125}I-IGF-II (●) and pouch young plasma (220 to 230 days, n = 2, pooled) incubated with ^{125}I-IGF-II (▲). Elution positions of molecular weight markers are indicated. G = gamma globulin, 160 kDa; B = BSA, 69 kDa; Ca = carbonic anhydrase, 30 kDa; Ct = chymotrypsinogen, 24 kDa; L = lysozyme, 14.3 kDa; I = ^{125}I-IGF, 7.5 kDa. Numbers 1 to 7 (D) indicate elution positions corresponding to lane numbers in Figure 7.3.

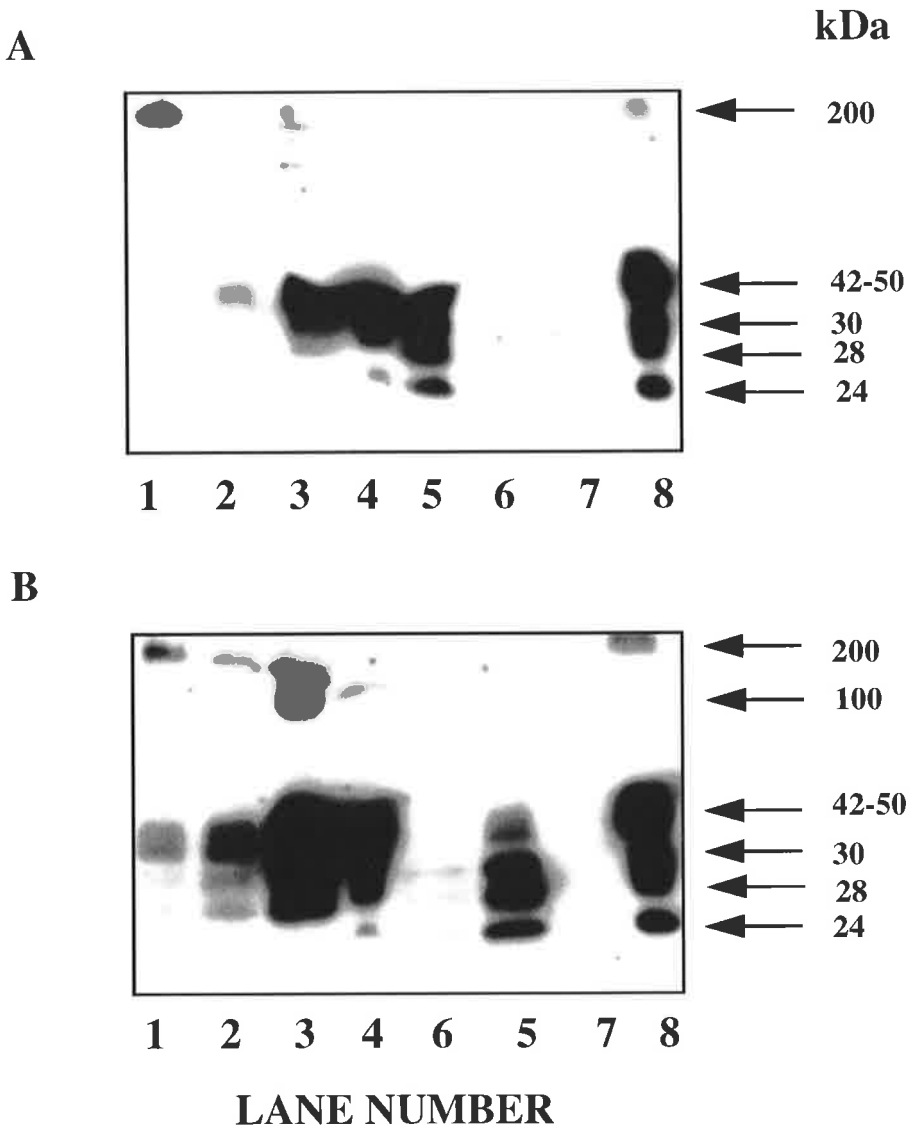


FIGURE 7.3 WESTERN LIGAND BLOT OF NEUTRAL GEL FILTRATION FRACTIONS. Fractions obtained after neutral gel filtration of plasma from the 50 day lactating wallaby mother (A) and 220 - 230 day wallaby pouch young (B) were dialysed, concentrated and the equivalent of half of each fraction subjected to SDS-PAGE and Western ligand blotting, as described in 7.2.3. Lane 1 = 12.3 - 13.2 ml; Lane 2 = 14.7 - 15 ml; Lane 3 = 15.9 - 16.2 ml; Lane 4 = 17.1 - 17.4 ml; Lane 5 = 18 - 18.6 ml; Lane 6 = 19.5 - 19.8 ml; Lane 7 = 22.2 - 22.5 ml elution volume; Lane 8 = normal ewe plasma (2 µl). Corresponding positions of lane numbers on the neutral gel filtration profile are indicated in Figure 7.2. Calculated molecular weights of IGFBP bands are indicated.

7.1, elute in the 25 to 70 kDa binding region under neutral gel filtration conditions. An unidentified 100 kDa band appears prominent in this experiment (Figure 7.3), although it is not present in the same plasma samples directly subjected to Western ligand blotting and may thus represent a proteolytic fragment of the 200 kDa band (Figure 7.5B, Lanes 9 and 10).

Northern Analysis of total liver RNA from wallabies indicates that transcripts for IGFBP-1, IGFBP-2 and IGFBP-4 are present in this species (Figure 7.4). The mRNA transcript for IGFBP-1 was approximately 1.8 kb, the same as that observed for rat and ovine IGFBP-1 (Figure 7.4A), although two smaller transcripts also appear to be present. IGFBP-1 mRNA was convincingly shown only in the pouch young subjected to extended phenobarbitone exposure (#181). This correlates with a strongly induced IGFBP band in the 30 kDa region in plasma from this animal collected by cutting the jugular vein and subjected to Western ligand blotting, suggesting the 30 kDa IGFBP may represent IGFBP-1 (Figure 7.5B). An mRNA transcript for IGFBP-2 was detected at approximately 1.6 kb in the wallaby, again as observed for sheep (Figure 7.4B). A weak band at approximately 3.2 kb was detected for wallaby IGFBP-4, while mRNA transcripts of approximately 2.6 kb were observed for sheep and rat, and two additional smaller molecular weight bands of approximately 2.1 and 1.8 kb in the sheep (Figure 7.4C). The hybridisations with ovine RNA shown in Figure 7.4B and 7.4C appear overexposed, due to the low stringency washes and long exposure times used to maximise detection of rat and wallaby IGFBP-2 and IGFBP-4 transcripts. IGFBP-3 mRNA transcripts could not be detected in total liver RNA from wallabies using a rat IGFBP-3 cDNA probe.

7.3.2 DEVELOPMENTAL CHANGES IN WALLABY IGFBPs

Plasma from wallaby mothers and pouch young from 10 days to 230 days lactation were examined by Western ligand blotting (Figure 7.5). The five molecular weight species identified in initial analyses were present in all samples. Circulating IGFBPs did not change throughout lactation in the wallaby mother (Figure 7.5A). There is an increase in circulating IGFBPs between 10 and 50 days and a further increase after 100 days of

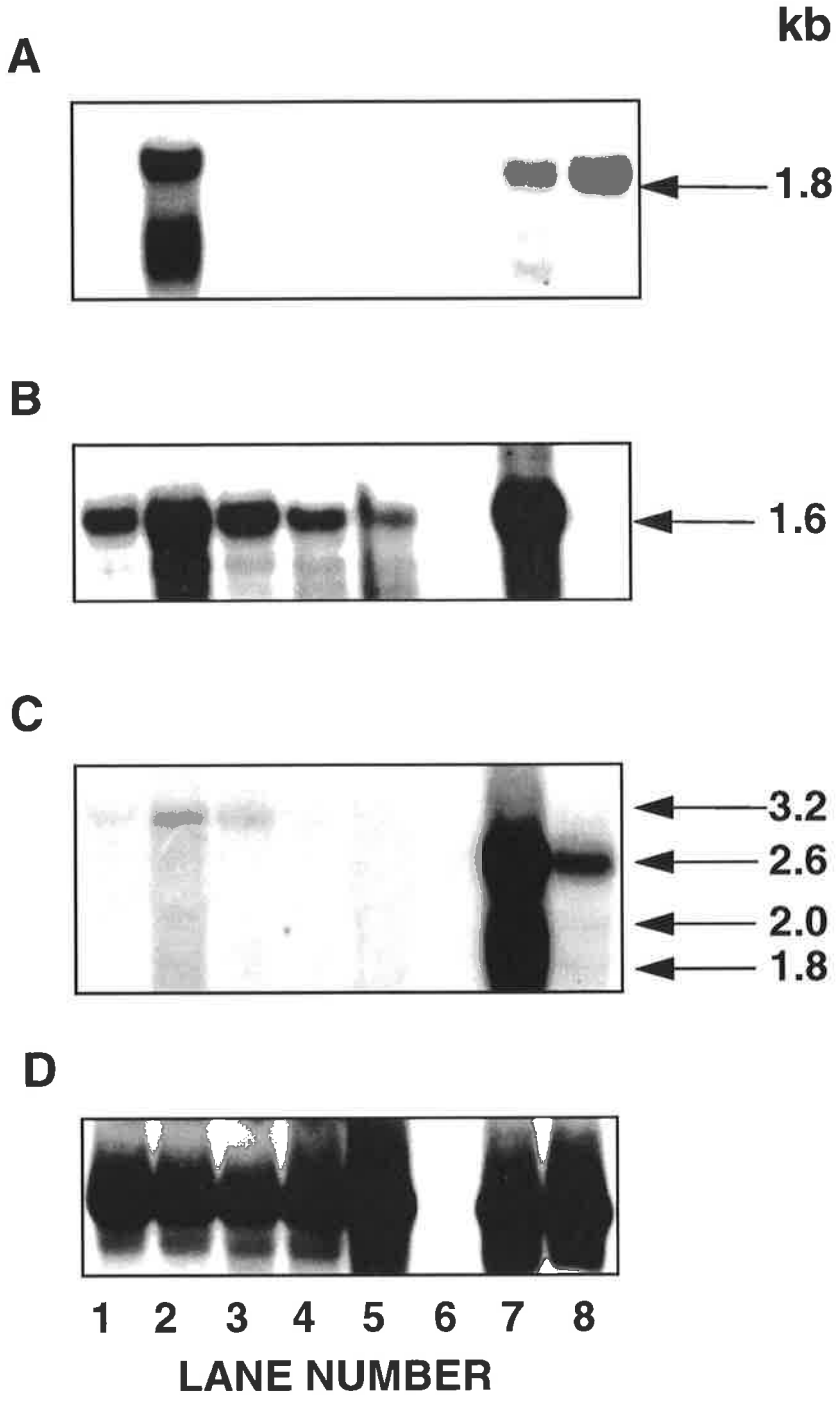


FIGURE 7.4 NORTHERN ANALYSIS OF WALLABY mRNA. Total liver RNA (40 µg) was subjected to Northern analysis, as described in 7.2.5. Filters were sequentially probed with A = rat IGFBP-1 (Murphy *et al.*, 1990), B = bovine IGFBP-2 (Upton *et al.*, 1990), C = ovine IGFBP-4 (Carr *et al.*, 1994a, Chapter 3) and D = 18S rRNA (Katz *et al.*, 1983). Lane 1 = 10 day wallaby pool; Lane 2 = #181, 50 day (extended phenobarbitone exposure); Lane 3 = #378, 50 day; Lane 4 = #375, 100 day; Lane 5 = #379, 100 day wallaby; Lane 6 = blank; Lane 7 = normal adult ewe liver; Lane 8 = normal adult rat liver. Sizes of mRNA transcripts, as determined from the migration of an RNA ladder, are indicated.

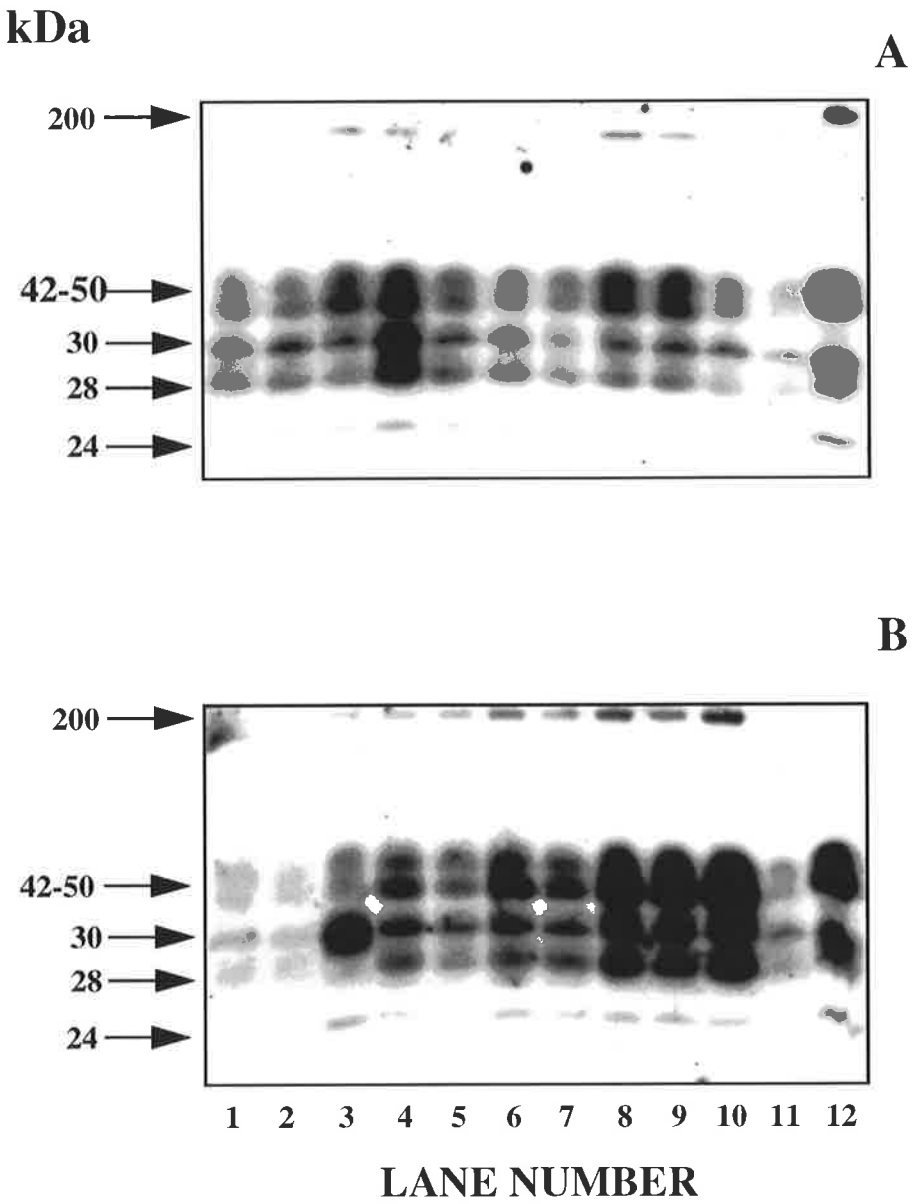


FIGURE 7.5 DEVELOPMENTAL ANALYSIS OF CIRCULATING IGFBPs. Wallaby plasma (2 µl) from 10 to 230 days lactation was subjected to Western ligand blotting, as described in 7.2.3. A = plasma from lactating mothers; B = plasma from their respective pouch young. Lane 1 = #125, 10 days; Lane 2 = #177, 10 days; Lane 3 = #181, 50 days (extended phenobarbitone exposure, jugular plasma collection); Lane 4 = #125, 50 days; Lane 5 = #378, 50 days; Lane 6 = #379, 100 days; Lane 7 = #375, 100 days; Lane 8 = #396, 185 days; Lane 9 = #352, 222 days; Lane 10 = #346, 230 days; Lane 11 = non-reproductive adult female; Lane 12 = normal ewe plasma. Calculated molecular weights of IGFBP bands are indicated.

suckling in the pouch young (Figure 7.5B). The changes in IGFBP levels in the pouch young were quantitated by laser densitometry (Figure 7.6). All IGFBP species increased with advancing age (p= 0.004, p=,0.01, p=0.005 and p=0.003, respectively for the 42 to 50, 30, 28 and 24 kDa IGFBPs).

7.4 DISCUSSION

The aims of this study were to characterise the IGFBPs in a species where early growth and development are very different to eutherian mammals in order to further investigate the roles of IGFs and IGFBPs in these processes. Western ligand blotting has shown that IGFBPs of approximately 42 to 50 kDa, 30 kDa, 28 kDa, 24 kDa and a high molecular weight band of approximately 200 kDa are present in wallaby plasma. These molecular weights are similar to those observed for other mammals (Rechler, 1993; Baxter, 1993). Note that the IGFBP band in wallaby plasma, sized at approximately 30 kDa, essentially co-migrates with the band identified as IGFBP-2 in sheep plasma, and sized at approximately 33 kDa in Chapter 5. This apparent discrepancy reflects the accuracy of molecular weight determination by this method. Neutral gel filtration and Western ligand blotting indicated the presence of a putative circulating type II IGF/cation-independent mannose 6-phosphate receptor in wallaby plasma. The circulating cation-independent mannose 6-phosphate receptor has been shown to lack the ability to bind IGF-II in chickens (Clairmont and Czech, 1989; Yang *et al.*, 1991). Therefore, this IGF-II binding ability appears to have evolved after the divergence of the reptilian ancestors of avian and mammalian lineages, but before the division of marsupials and eutherian mammals. Neutral gel filtration suggests another high molecular weight species (70 to 160 kDa) in adult wallabies, mothers and pouch young. This binding species was shown to contain primarily the 42 to 50 kDa doublet after SDS-PAGE and Western ligand blotting. Thus, this high molecular weight binding species may be analogous to the ternary complex observed in other eutherian mammals, comprising IGFBP-3, IGF and an acid labile non-IGF binding subunit (ALS) (1.2.2.3 and 1.2.2.7).

Although immunoblotting failed to identify the circulating IGFBPs, Northern

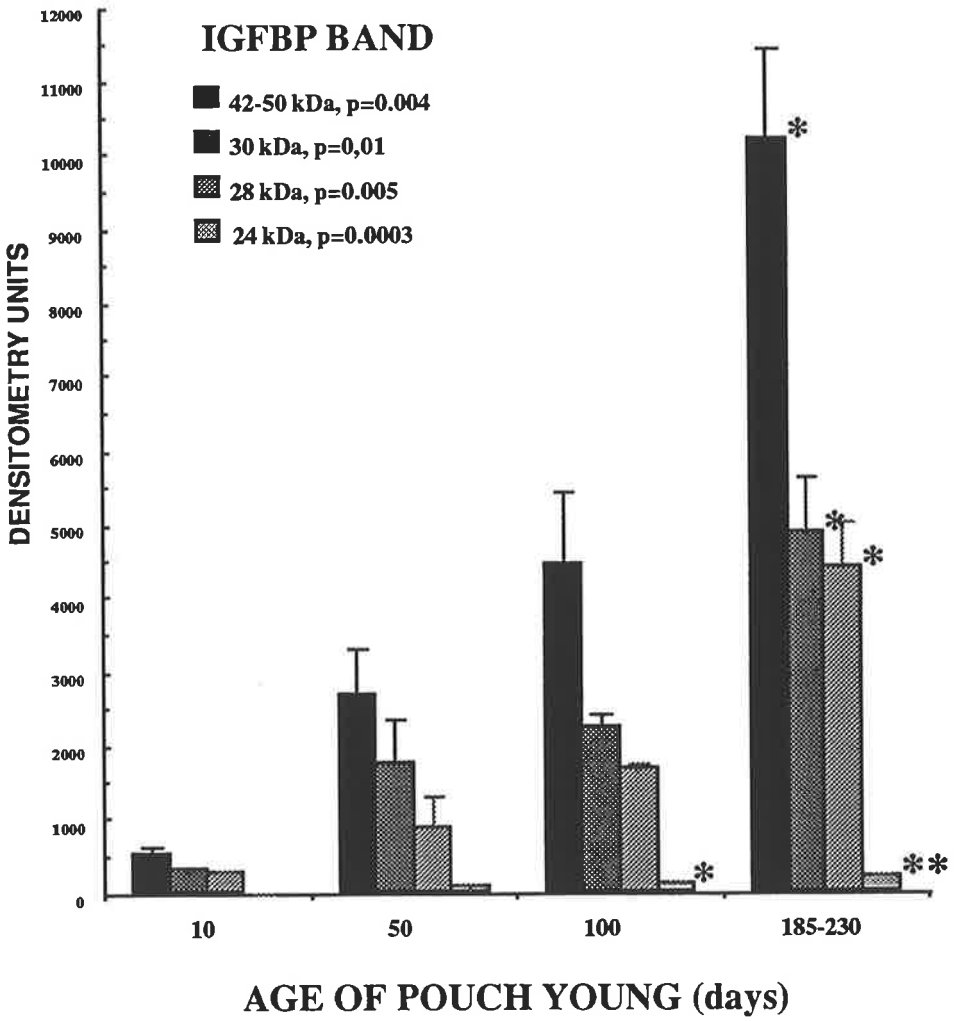


FIGURE 7.6 QUANTITATION OF DEVELOPMENTAL CHANGES IN WALLABY IGFBPs. Lanes 1 to 10 of the Western ligand blot shown in Figure 7.5B were quantitated by laser densitometry and densities expressed as arbitrary densitometry units. Values for pouch young at 10 days (n = 2); 50 days (n = 2, excluding #181); 100 days (n = 2); and 185 to 230 days (n = 3); for the 24, 28, 30 and 42 to 50 kDa bands were subjected to ANOVA with specific Bonferroni contrasts (Proc GLM, SAS) (2.2.5). * = significantly different in comparison to 10 day pouch young values for the same IGFBP. p values for ANOVA are indicated. A significant increase in the levels of all IGFBPs with increasing age was also indicated by Linear regression analysis (data not shown).

analysis of total liver RNA showed transcripts for IGFBP-1, IGFBP-2 and IGFBP-4, suggesting the presence of homologues for these IGFBPs in the wallaby. IGFBP-1 mRNA was barely detectable in pouch young liver RNA. However, liver RNA from a pouch young kept under anaesthesia during lung perfusion, prior to removal of the liver, contained readily detectable levels of IGFBP-1 mRNA. Anaesthesia has been reported to rapidly stimulate IGFBP-1 levels in sheep (Lord *et al.*, 1994). Thus, although not representing a normal 50 day pouch young, tissues from this animal were useful in identifying an IGFBP-1 mRNA transcript in the wallaby. Additionally, this animal had high levels of the 30 kDa IGFBP in plasma, which was collected by cutting the jugular, compared to other pouch young of similar age. The IGFBP-2 mRNA transcript in this animal was also highly abundant. Thus elevation of the 30 kDa circulating IGFBP may result from a very rapid translation of the elevated mRNAs for IGFBP-1 and IGFBP-2, or perhaps from contamination of blood samples (due to the collection method) by extracellular fluid or lymph, which in the sheep contains IGFBPs of this size (Lord *et al.*, 1991).

IGFBP-5 and IGFBP-6 were not examined in this study. IGFBP-3 mRNA could not be detected with a rat IGFBP-3 probe. However, we have also experienced difficulty in detecting ovine IGFBP-3 from total RNA with this probe probably due to problems with cross-species detection using heterologous probes rather than a lack of IGFBP-3 mRNA in the wallaby. With the exception of IGFBP-4, the sizes of the mRNA transcripts in the wallaby were comparable to those of sheep and rat: (1.8 kb, IGFBP-1; 1.6 kb, IGFBP-2). The transcript identified as wallaby IGFBP-4 is larger than that observed for rat and sheep (2.6, sheep and rat; 3.2 kb wallaby) and appears to be present in the liver of the early lactation pouch young at very low levels. This may be due to low expression of IGFBP-4 mRNA in the wallaby or substantial divergence of wallaby IGFBP-4 DNA sequence from that of sheep, leading to poor hybridisation of the oIGFBP-4 DNA probe. Considering the high cross species conservation of IGFBP-4 sequences indicated in Chapter 3, the IGFBP-4 cDNA sequence from the wallaby may prove an interesting comparison to delineate conserved structural and regulatory regions of IGFBP-4.

A preliminary assessment of developmental changes in circulating IGFBPs indicates

that in the pouch young there is a general increase in the levels of most IGFBPs with increasing age, but no major changes in circulating IGFBPs in the lactating mother. The timing of the rise in circulating IGFBPs in the pouch young may be related to the acceleration of growth rate and maturation of the pouch young, which occurs after approximately 100 days or the commencement of grazing activity (180 to 200 days) (Tyndale-Biscoe and Janssens, 1988). Events such as the change in nutrition, or the onset of growth hormone (GH) responsiveness stimulate alterations in IGFBPs in eutherian mammals (1.2.5) and thus are possible stimuli for the changes in circulating IGFBPs in the wallaby pouch young.

In conclusion, in terms of the types of IGFBPs present and their circulating forms, the wallaby, *M. eugenii*, appears to be similar to other mammals. Together with their developmental differences and the accessibility of the growing pouch young, the marsupial may provide a useful model for comparative studies to further elucidate the roles and regulation of IGFs and IGFBPs in mammalian growth and development. The marsupial pouch young undergoes substantial changes in circulating IGFBPs throughout the suckling period for which the physical or endocrine stimuli remain to be investigated.

CHAPTER 8. GENERAL DISCUSSION

The main focus of the studies described in this thesis were to investigate the ontogeny of IGFBPs in the sheep, in normal and growth restricted fetuses, to further our understanding of the roles and regulation of IGFBPs throughout fetal growth and development. Specifically, one initial aim was to characterise ovine IGFBP-4 (oIGFBP-4), a novel IGFBP at the commencement of the project, and to generate valuable molecular tools for further investigations of the function and regulation of IGFBP-4 in the sheep fetus.

These studies have extended our knowledge of the ontogeny of circulating IGFBPs and tissue mRNA abundance for IGFBP-2 and IGFBP-4 in major fetal organs, over a wide time frame of gestation, in a large mammalian species. Circulating changes in IGFBPs were identified and found to relate to fetal age, circulating IGFs and fetal growth status and potential tissue sources of circulating IGFBP-2 and IGFBP-4 were also identified. The results for oIGFBP-4 are particularly novel since the physiology of this relatively new IGFBP is largely undefined.

Determination of ovine IGFBP-4 cDNA sequence

The cDNA sequence and ontogeny of oIGFBP-4, was isolated and characterised. N-terminal protein sequence data for oIGFBP-4 and recently published cDNA sequences for human and rat IGFBP-4 were used to design primers for PCR amplification of a small N-terminal region from oIGFBP-4 cDNA. This was unsuccessful for oIGFBP-4, although an N-terminal IGFBP-4 product was generated from rat cDNA and subsequently used as a probe for cDNA library screening. Ovine liver cDNA library screening with this probe was also unsuccessful, although a near full length human IGFBP-4 cDNA was obtained from a human fetal liver library. This human IGFBP-4 cDNA clone was then used for further liver cDNA library screening for oIGFBP-4, resulting in the isolation of partial oIGFBP-4 cDNA clones, lacking N-terminal coding and 5' untranslated sequences. The N-terminal sequences encoding mature oIGFBP-4 were isolated by reverse transcription polymerase chain

reaction (RT-PCR). The DNA and translated protein sequence for oIGFBP-4 showed strong identity with IGFBP-4 sequences from human, rat and, in particular, the cow (Shimasaki *et al.*, 1990a; Moser *et al.*, 1992). The DNA sequence over the protein coding region of ovine and bovine IGFBP-4 is 98 % identical, while the mature protein contains only three amino acid substitutions, suggesting strong evolutionary conservation of protein sequence and hence structure and function. Additionally, the 1000 bp of 3' non-translated sequence from ovine and bovine IGFBP-4 is 96 % identical, with two DNA insertions of 79 and seven bp in the ovine IGFBP-4 3' end, implying conservation of 3' regulatory sequences. Characterisation of the size and distribution of oIGFBP-4 mRNA transcripts in the sheep indicated the presence of one major and two minor mRNA species of approximately 2.6, 2.1 and 1.8 kb, respectively. IGFBP-4 mRNA levels varied in a tissue specific and developmental manner, with highest levels in the liver ≥ kidney > lung >> heart of the adult, while IGFBP-4 mRNA was readily detectable in the early fetal ovine heart. These observations are the first descriptions of the tissue distribution and developmental regulation of IGFBP-4 mRNA, in a large mammalian species, and were further investigated.

Generation of specific ovine IGFBP-4 antibodies

In addition to generating the ability to characterise IGFBP-4 at the mRNA level, these studies also aimed to develop antibodies to IGFBP-4 to investigate IGFBP-4 protein. Collaborators P. E. Walton and P. A. Grant, CRC for Tissue Growth and Repair, had previously isolated and characterised oIGFBP-4 from sheep plasma and generated oIGFBP-4 antibodies with which to develop a specific IGFBP-4 RIA. However, similar to other commercially available human IGFBP-4 antibodies, the IGFBP-4 antibodies generated showed marked cross-reactivity with oIGFBP-2. This thesis has described the development of a specific oIGFBP-4 antibody, generated by immunising with peptide regions of low homology between the different IGFBPs, especially IGFBP-2 and IGFBP-4. As an alternative to peptide conjugation, which is traditionally used to enhance the immunogenicity of peptides, these studies used a novel hybrid bacteriophage display system

(Greenwood *et al.*, 1991). This system involved generating hybrid bacteriophage particles that contained the peptide region of interest incorporated within the outer coat protein shell of a bacteriophage, thus presenting the peptide to the immune system. DNA constructs were generated encoding two different 11 amino acid regions of IGFBP-4 linked to the mature N-terminus of the major coat protein for the bacteriophage, fd. Peptide - coat protein fusion molecules were successfully incorporated into hybrid bacteriophage particles for both of the selected peptide regions, and used to directly immunise rabbits. The bacteriophage preparation proved to be a good antigen, producing anti-fd antibodies by the second boost with or with out adjuvant. Using this hybrid bacteriophage display system, an antibody, specific for oIGFBP-4 that did not cross react with other IGFBPs, was generated. The apparent titre of this antibody appeared low, although this may be a result of the method of titration. Although further experiments were not performed with this antibody, it may be useful for developing a specific oIGFBP-4 RIA and for functional studies of oIGFBP-4. The general method of hybrid bacteriophage display for peptide immunisations is relatively simple and the DNA construct provides a replentishable source of antigen for further immunisations. This technique may be useful for generating a panel of antibodies against different regions of IGFBPs, to investigate structure / function relationships, such as determining the IGF binding site, or for the development of antibodies against other IGFBPs or IGFBPs from different species, for which large quantities of protein are not readily available.

The availability of antibodies and DNA probes for oIGFBP-4 should enable us to further characterise this IGFBP at both the level of the protein and gene, in different physiological states in the sheep, a mammalian species whose physiology and endocrinology have been extensively characterised throughout development. The latter chapters of this thesis describe the first of such studies which has demonstrated developmental and growth related alterations in several IGFBPs, including IGFBP-4, in the ovine fetus.

IGFBPs in fetal growth and development

Circulating IGFBP-2 rose in early to mid gestation, then declined between late gestation and 1 day post-natally. These changes were paralleled by liver and kidney IGFBP-2 mRNA, suggesting these tissues to be the primary sources of circulating IGFBP-2. Although detectable in early gestation, IGFBP-2 mRNA was not present at significant levels in the heart or lung at later gestational ages, indicating strict developmental regulation of IGFBP-2 mRNA in these tissues. Circulating changes in IGFBP-2 were paralleled by alterations in circulating IGF-II. In the growth restricted fetus, the ontogeny of circulating IGFBP-2 was altered, with prematurely elevated levels in mid gestation, prior to the onset of fetal growth retardation, and an early decline in circulating IGFBP-2 in late gestation. These changes in circulating IGFBP-2 were negatively associated with fetal weights and weights of some fetal organs and thus IGFBP-2 may be involved in the observed inhibition of fetal growth. The changes in circulating IGFBP-2 in the growth restricted fetus again paralleled previously reported alterations in the levels of circulating IGF-II, which are elevated in the early stages of late gestation (Jones *et al.*, 1988), then decline later at 128 days gestation (Owens *et al.*, 1994), when IGFBP-2 levels are also decreasing. These results suggest that the circulating ontogenic changes in IGFBP-2 may be mediated by alterations in IGF-II, or an upstream factor common to regulation of both IGF-II and IGFBP-2. The decline in hepatic IGF-II mRNA in the late gestation fetal sheep has been suggested to be negatively related to the pre-partum cortisol surge (Li *et al.*, 1993). Thus, the late ontogeny of IGFBP-2 may also relate, either directly or indirectly via IGF-II, to changes in fetal cortisol levels, as is illustrated in Figure 8.1. In the growth restricted fetus, there are many changes in endocrine factors known to regulate IGFBP-2 post-natally, which may therefore mediate the observed alterations in IGFBP-2. Growth restriction, induced by restriction of placental substrate supply, is characterised by hypoglycaemia and hypoinsulinaemia and prematurely elevated cortisol levels in late gestation (Robinson *et al.*, 1994). Low insulin or glucose stimulates IGFBP-2 levels post-natally (Ooi *et al.*, 1992, Rechler, 1993), while prematurely elevated cortisol levels, as proposed above, may lead to

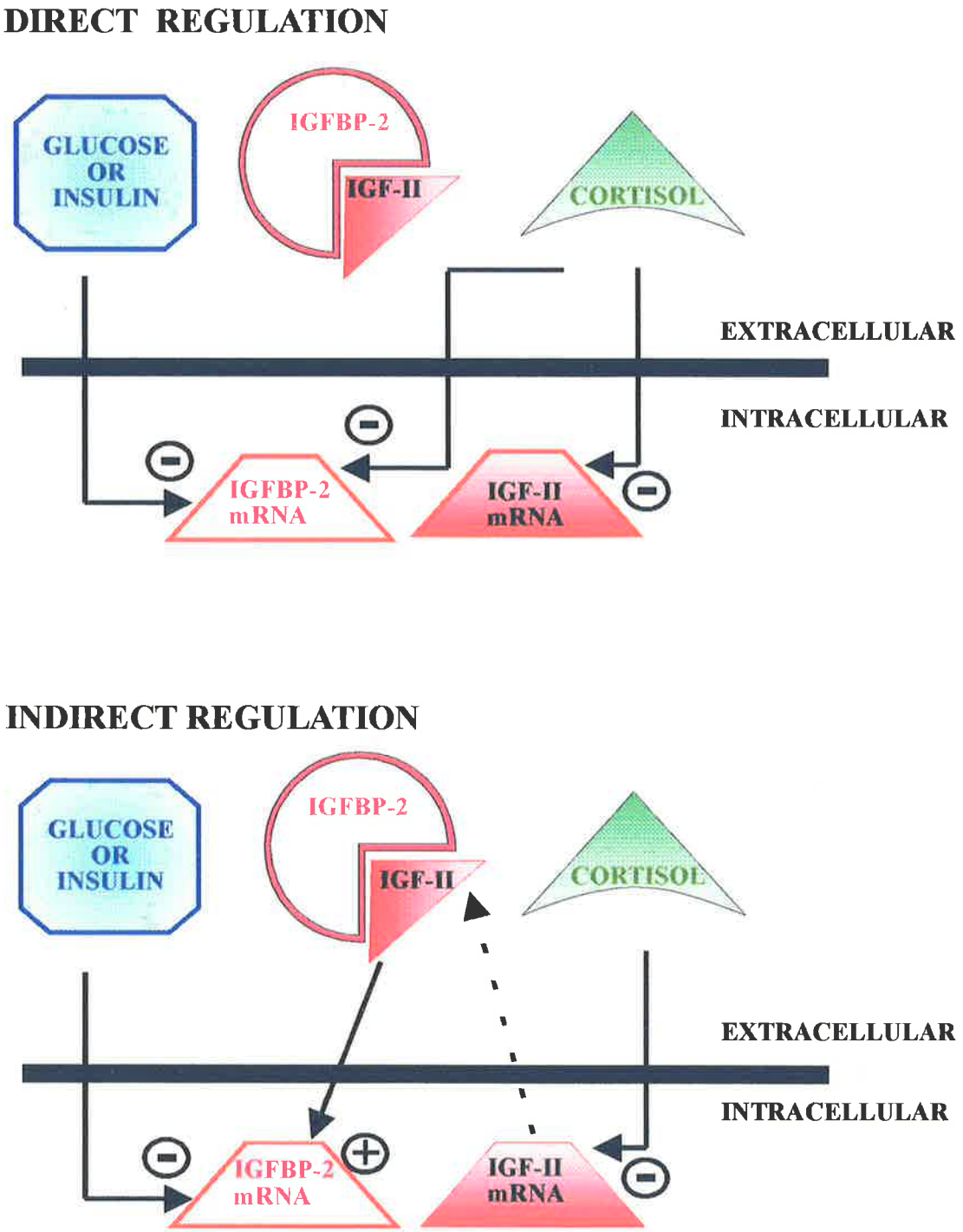


FIGURE 8.1 PROPOSED REGULATION OF IGFBP-2 *IN UTERO*. IGFBP-2 is negatively regulated by circulating glucose or insulin at the level of IGFBP-2 mRNA (either indirectly or directly). Two alternative mechanisms for cortisol regulation of IGFBP-2 are shown. Cortisol may directly regulate IGFBP-2 via an inhibitory effect on IGFBP-2 mRNA (upper panel). Alternatively, cortisol may indirectly regulate IGFBP-2 through a reduction in IGF-II mRNA and subsequently IGF-II protein. IGF-II is a proposed positive stimulus for IGFBP-2 mRNA and protein. Thus, a reduction in IGF-II induced by cortisol results in a decrease in circulating IGFBP-2 and IGFBP-2 tissue mRNA.

an early decline in IGFBP-2, either directly or through an early reduction in IGF-II levels (Li *et al.*, 1993). These hypothesised interactions are schematically illustrated in Figure 8.1. Thus, a complex interaction of factors may be responsible for both the normal ontogeny of IGFBP-2 and responses of IGFBP-2 to perturbation.

Circulating IGFBP-3 and IGFBP-4 increased as gestation progressed in the normal fetal sheep. Circulating IGFBP-4 was positively correlated with hepatic IGFBP-4 mRNA, suggesting that the liver may be the primary contributor to circulating IGFBP-4 levels. IGFBP-4 mRNA was detectable in the fetal heart in early gestation, but not at later ages, similar to the ontogeny of IGFBP-2 mRNA in the heart. In contrast to IGFBP-2 mRNA in the lung, IGFBP-4 mRNA was present at high levels in this tissue throughout the entire gestational period. These observations suggest both co-ordinate and independent regulation of IGFBP-2 and IGFBP-4 mRNA in a highly tissue specific manner.

Circulating IGFBP-3 and IGFBP-4 were correlated with circulating IGF-I in the normal fetus, suggesting a specific link between IGFBP-3, IGFBP-4 and IGF-I. Circulating IGFBP-4 also increased with gestational age in the growth restricted fetus, and by late gestation IGFBP-4 levels were elevated in the growth restricted fetuses compared to controls. Restriction of fetal growth did not alter circulating IGFBP-3 levels although they tended to be lower in late gestation compared to controls. Circulating IGFBP-3 and IGFBP-4 correlated with fetal weight in the normal fetus and fetal body weight or the weight of specific organs in the growth restricted fetus, suggesting positive influences of these proteins on growth *in utero*. Thus, the observed elevation of circulating IGFBP-4 levels in the late gestation growth restricted fetus may not be inhibitory to fetal growth but may act to enhance the tissue delivery of circulating IGF and promote IGF actions, although relative to control fetuses growth is retarded due to the lower abundance of circulating IGF (Owens *et al.*, 1994). The changes in IGFBP-3 and IGFBP-4 in the growth restricted fetus parallel a reported decline in circulating fetal IGF-I levels (Owens *et al.*, 1994). Thus, although IGF-I may be associated with IGFBP-3 and IGFBP-4 in the normal fetus, these factors, particularly IGF-I and IGFBP-4, are not co-ordinated in the late gestation growth

restricted fetus. Since the *in vivo* regulation of IGFBP-4 is largely unknown, there is little basis for speculation on possible mediators of these changes in IGFBP-4. However, there are several *in vitro* reports of IGF stimulated IGFBP-4 protease activity (Neely and Rosenfeld, 1992; Kanzaki *et al.*, 1994) (1.2.5.2). Thus the decline in IGF-I may lead to enhanced stability of IGFBP-4, through reduced proteolytic activity following restriction of fetal growth. Alternatively, changes in fetal hormones such as progesterone may alter IGFBP-4 gene expression through interaction with a homologous promoter element in the sheep gene to that proposed for the rat (Gao *et al.*, 1993).

Hypothesised relationships between IGFBPs and fetal growth and development

The observed changes in IGFs and IGFBPs throughout gestation, may relate to developmental changes in the growth characteristics of the developing fetus. Circulating levels of IGF-I and IGF-II and IGFBPs are substantial in the early gestation ovine fetus, suggesting that IGFs and IGFBPs may have an endocrine function in early development. Additionally, the high levels of IGFBP-2 and IGFBP-4 mRNAs in the heart and lung during early development are suggestive of important paracrine or autocrine actions of IGFBP-2 and -4 within these tissues at this early stage. In early to mid gestation, when cell differentiation and determination of organ morphogenesis is important, circulating IGF-II and IGFBP-2 are elevated. These factors may act to co-ordinate growth, development and differentiation of various tissues and organs. This is supported by the lack of correlation of circulating IGF-II levels with fetal weight, which is observed in many studies (Han and Hill, 1994). In contrast, circulating IGF-I, IGFBP-3 and IGFBP-4 increase throughout gestation and correlate positively with fetal weight. Thus, IGF-I / IGFBP-3 or IGFBP-4 complexes may be involved in co-ordinating or controlling fetal body and organ growth and weight gain in a positive fashion, as occurs post-natally. These hypothesised roles for IGFs and IGFBPs in normal fetal growth and development are illustrated in Figure 8.2.

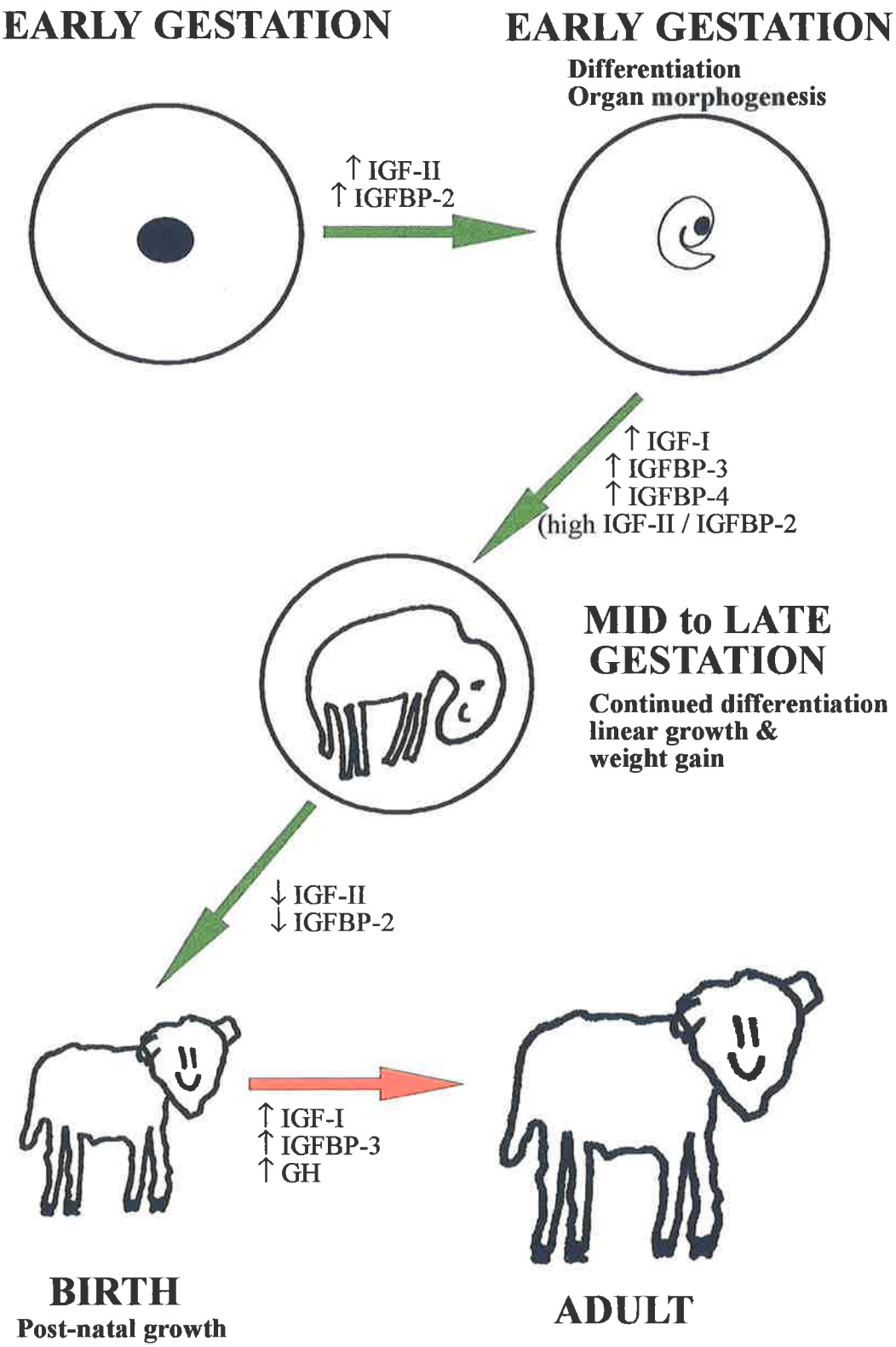


FIGURE 8.2 HYPOTHESISED RELATIONSHIP OF IGFs AND IGFBPs TO GROWTH AND DEVELOPMENT. Increasing levels of IGF-II and IGFBP-2 in early development stimulate differentiation and growth of organs. Circulating IGF-II and IGFBP-2 remain high in mid to late gestation, promoting further organ growth and maturation. Increasing levels of circulating IGF-I, IGFBP-3 and IGFBP-4 in mid to late gestation promote linear fetal growth and weight gain. Circulating IGF-II and IGFBP-2 levels decline just prior to birth and IGF-I, IGFBP-3 and GH increase throughout post-natal life to promote growth.

One can also speculate that the alterations in IGFBPs may contribute to the phenotype of intra-uterine growth retardation (IUGR), as follows:

- structural alterations in some organs due to inappropriate morphological development directed by elevated IGF-II and IGFBP-2 in early gestation.
- asymmetric growth retardation through a redistribution of IGFs onto different IGFBPs (eg. IGFBP-2 in early to mid gestation, IGFBP-4, in late gestation), which targets IGF to different cells or tissues and may modify its bioactivity.
- immaturity of some organs or systems (eg. wool [Harding *et al.*, 1985], small intestine [Trahair *et al.*, 1993]), due to pre-maturely decreased levels of differentiative factors, such as IGF-II and IGFBP-2 either within the tissues or in the circulation.
- restriction of growth through decreased levels of IGFs and increased levels of some IGFBPs, particularly IGFBP-2, which is inversely associated with fetal body and organ weight.

These hypothesised alterations are depicted in Figure 8.3.

IGFBPs in the marsupial - a comparative developmental model

These studies of the IGF / IGFBP axes in the developing ovine fetus contrasts to observations in species such as the rat. For example, IGF-II in the rodent is fetal specific, with no detectable IGF-II in the adult rat circulation, while IGF-II is readily detectable in both the fetal and adult sheep, humans and other larger mammals (Cohick and Clemmons, 1993; Han and Hill, 1994; Chapter 5). The chicken lacks a functional type II IGF receptor (Yang *et al.*, 1991), which contrasts with the elevated levels of the circulating type II IGF receptor in the fetal sheep circulation compared to the adult (Butler and Gluckman, 1986) and the high abundance of the type II IGF receptor in fetal sheep liver (Owens *et al.*, 1980). Such comparative studies in different experimental systems may improve our understanding of the factors central to growth and development. This thesis also describes IGFBPs in a marsupial where, in contrast to the intra-uterine environment of other mammalian species or

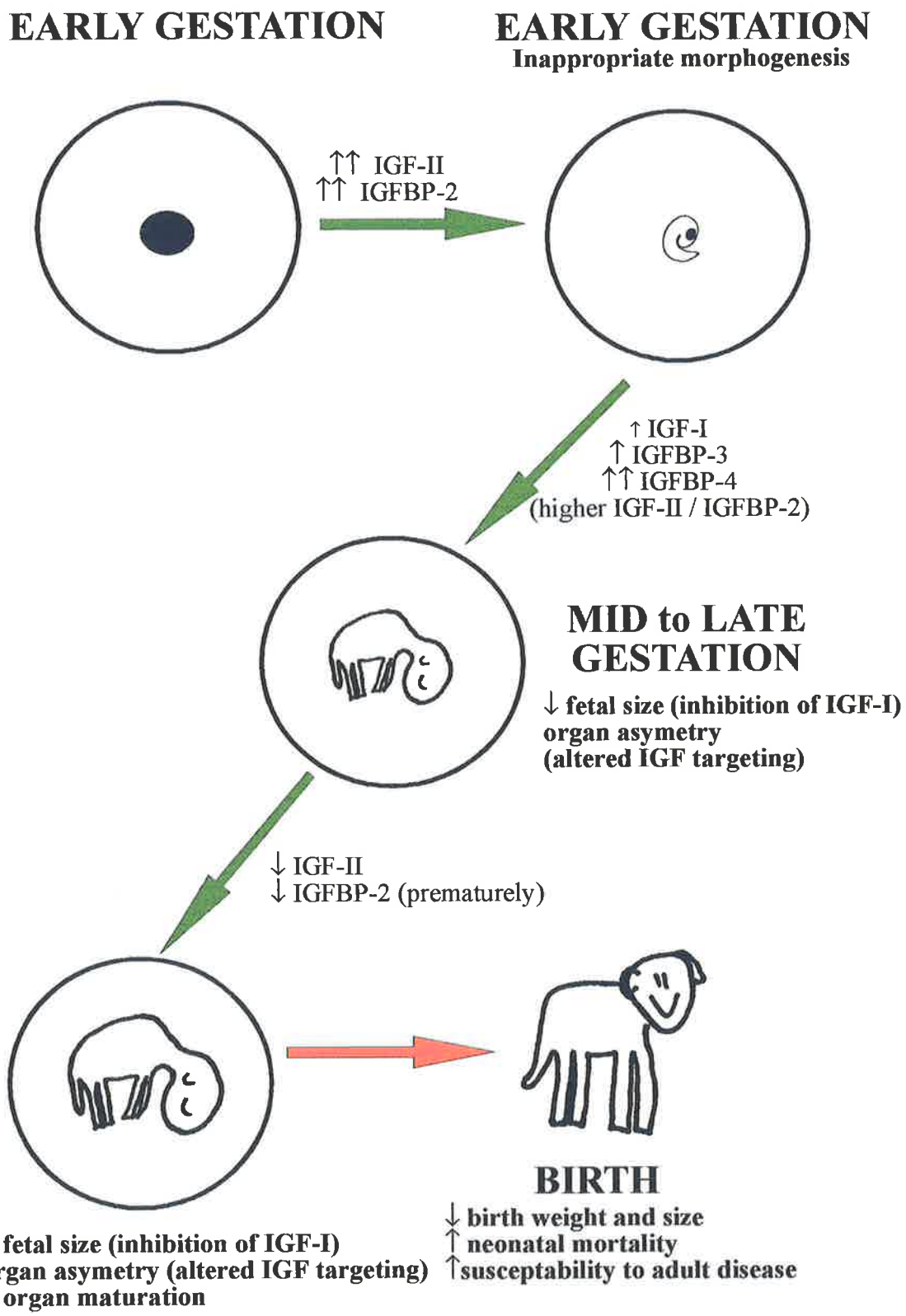


FIGURE 8.3 HYPOTHESISED INVOLVEMENT OF IGFs AND IGFBPs IN IUGR. IGF-II and IGFBP-2 are elevated very early in gestation leading to inappropriate organ morphogenesis. Maintainence of elevated IGF-II and IGFBP-2 throughout mid gestation, in combination with decreased IGF-I levels, leads to a reduction in fetal size due to reduced levels of IGF and inhibition of IGF bioactivity by high IGFBP-2, and asymetric organ growth due to altered tissue targeting of IGF by IGFBPs. The reduced levels of IGF-I continues to retard fetal growth while the premature decline in IGF-II and IGFBP-2 results in a lack of maturation of particular organs, resulting in a compromised neonate.

the *in ovo* environment of the avian lineages, growth and development of the marsupial primarily occurs in the pouch during the lactation period. IGFBPs were demonstrated to be present in both the lactating wallaby mother and pouch young throughout the lactation period (Chapter 7). These IGFBPs were similar in molecular size to those observed in the human and sheep. mRNA transcripts for IGFBP-1, IGFBP-2 and IGFBP-4 were demonstrated in the liver of wallaby pouch young, suggesting the presence of these IGFBP homologues in the wallaby. Additionally, the sizes of the mRNA transcripts for wallaby IGFBP-1 and IGFBP-2 were similar to those for the sheep and rat. Therefore, marsupial IGFBPs are very similar to those of other eutherian mammals. Circulating IGFBPs were unchanged throughout lactation in the wallaby mother, but showed a progressive increase in the wallaby pouch young with increasing age. The timing of these observed developmental changes in IGFBPs may relate to the growth rate and nutritional intake of the pouch young, which is yet to be investigated. These observed similarities in IGFBPs between eutherian mammals and marsupials, in combination with developmental differences and the relative ease of manipulations of the pouch young, in comparison to the fetus *in utero*, indicates that the wallaby may provide a useful experimental model for further investigations of the roles and regulators of IGFs and IGFBPs in growth and development.

In conclusion, this thesis has described the isolation and characterisation of oIGFBP-4 cDNA sequence and the generation of a specific oIGFBP-4 antibody. These tools were used to characterise oIGFBP-4 mRNA and may be used to further characterise IGFBP-4 protein. Studies have also demonstrated ontogenic and growth related changes in IGFBPs, normally and during growth restriction *in utero*, and have identified potential tissue sources of circulating IGFBP-2 and IGFBP-4, which may direct the endocrine functions of these proteins. My results also suggest specific associations of IGF-II with IGFBP-2, and IGF-I with IGFBP-3 and IGFBP-4, as well as negative and positive associations of IGFBP-2, IGFBP-3 and -4 with fetal growth, respectively. From these and previous studies it has been speculated that cortisol or IGF-II may be possible mediators of the ontogeny of IGFBP-2 (Figure 8.2) and that the IGFs and IGFBPs are involved in normal fetal growth

and development (Figure 8.2) and in the aetiology of IUGR (Figure 8.3). Finally, the IGFBPs have been characterised in a marsupial which may provide a useful comparative model for investigations of the endocrine and environmental stimuli that regulate IGF and IGFBPs throughout growth and development.

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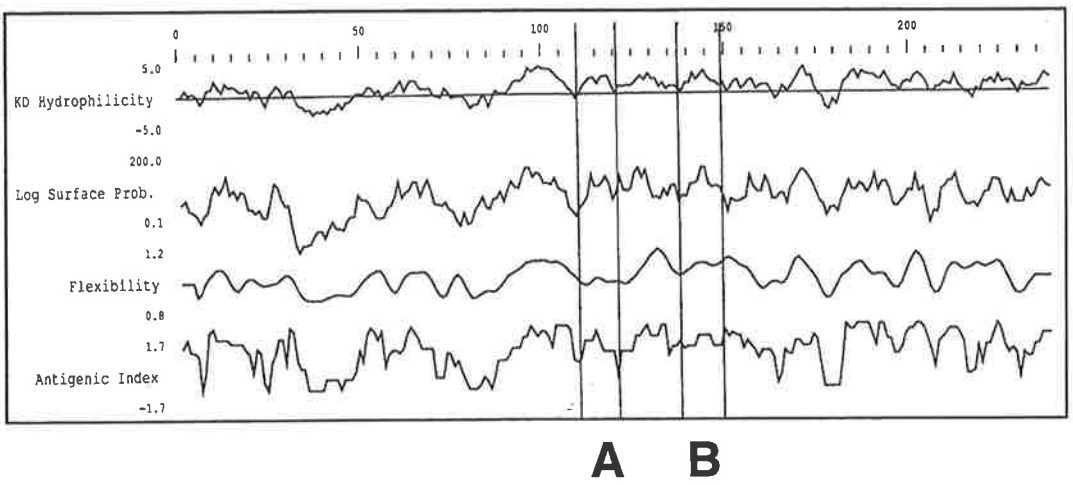
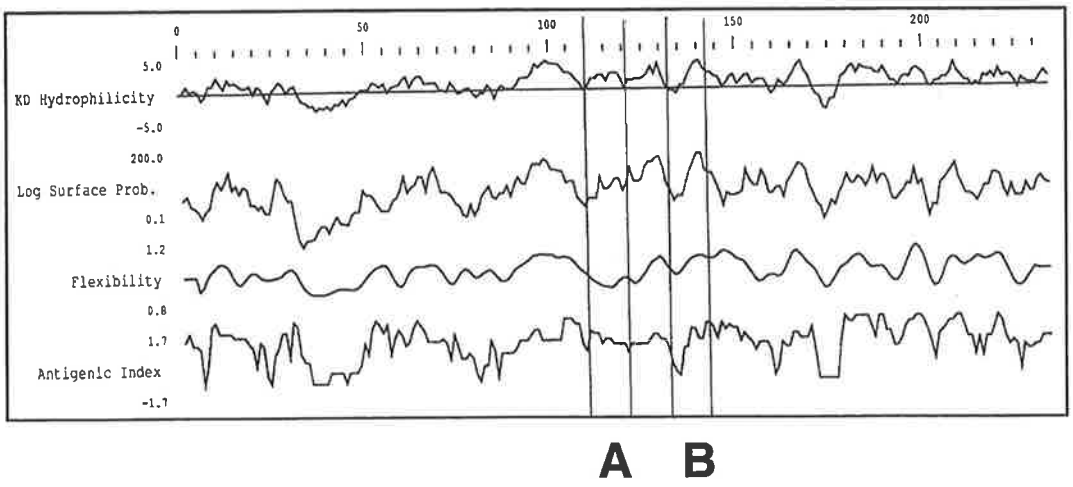
APPENDICES

1A = Human

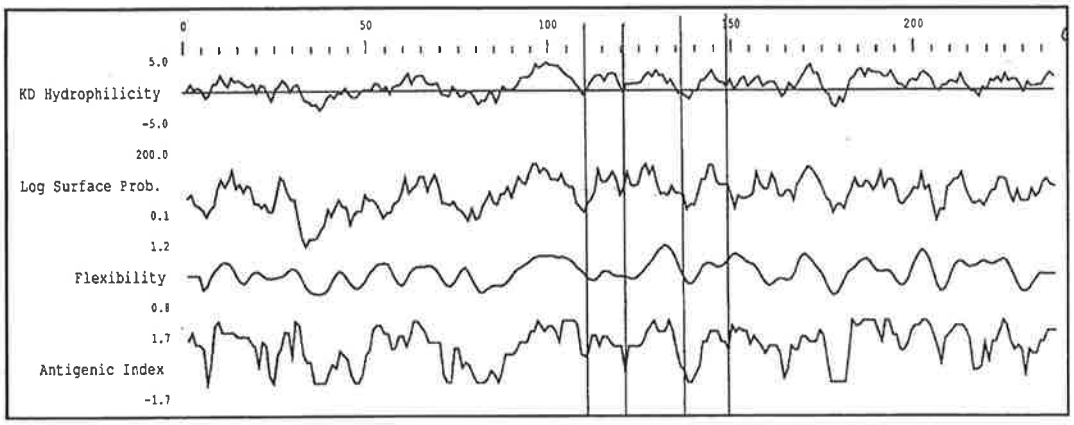


1B = Rat



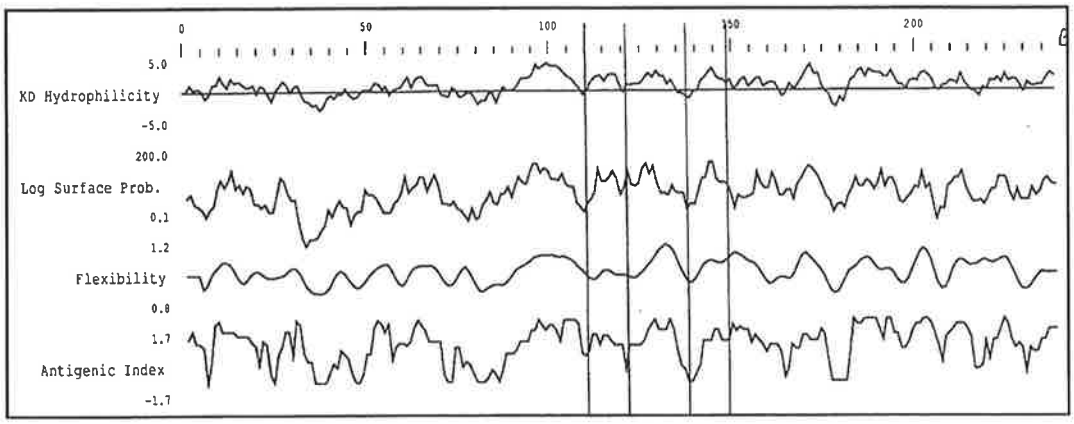
APPENDIX I. Antigenicity and hydrophilicity profiles of human (A), rat (B), ovine (C) and bovine (D) IGFBP-4, as determined by 1=Jameson and Wolf (1988) antigenic index, KD = Kyte and Doolittle, (1982), hydrophilicity profile. 2 = Wellings *et al.* (1985) antigenicity determination. 3 = Hopp and Woods (1981), hydrophilicity profile. The position of peptide regions A and B are indicated.

1C = Ovine



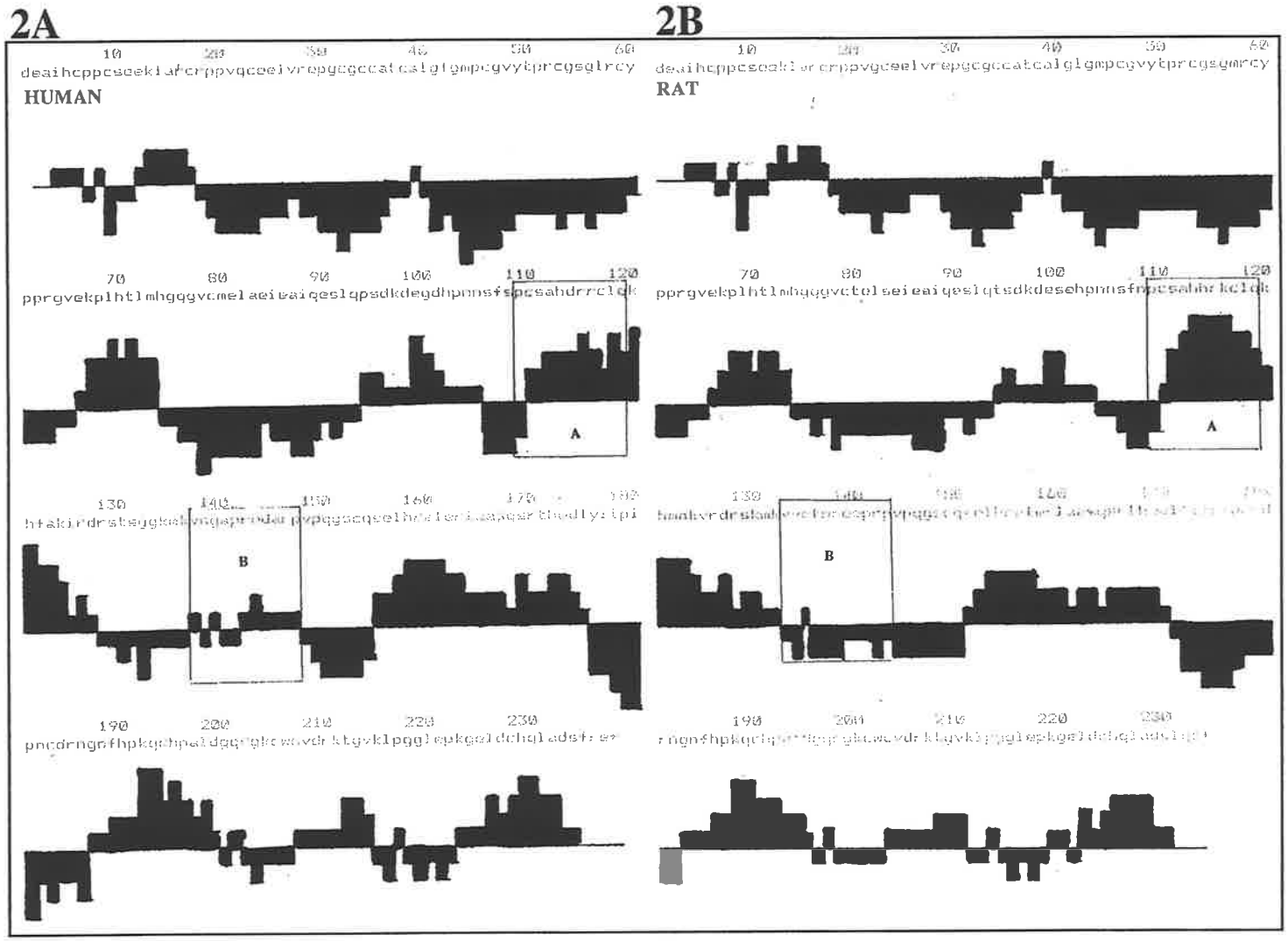
A B

1D = Bovine

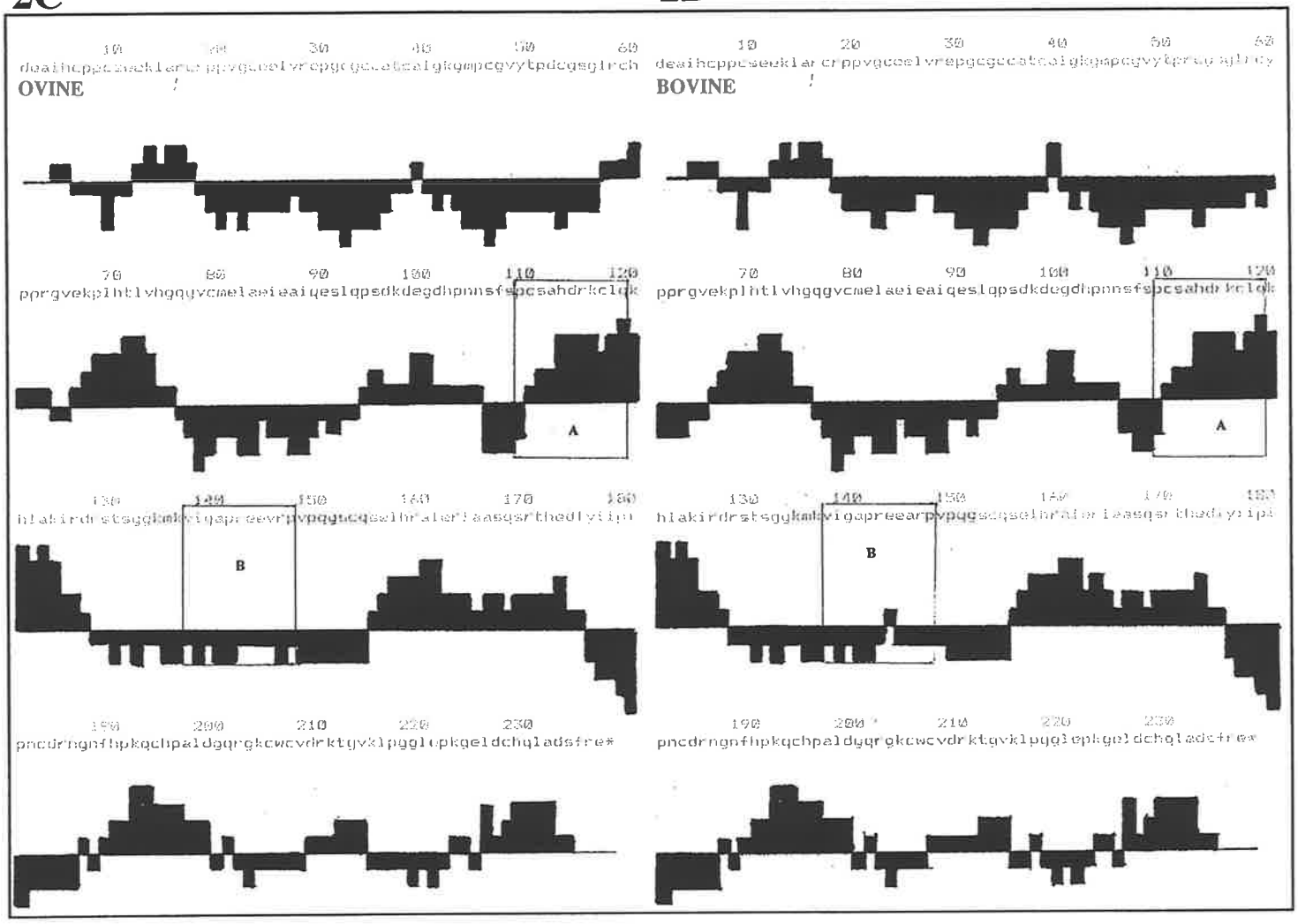


A B

APPENDIX I (contiuned - Jameson and Wolf, [1988], antigenic index, Kyte and Doolittle [1982], hydrophilicity)



APPENDIX I (continued - Wellings *et al.*, 1985, antigenicity)



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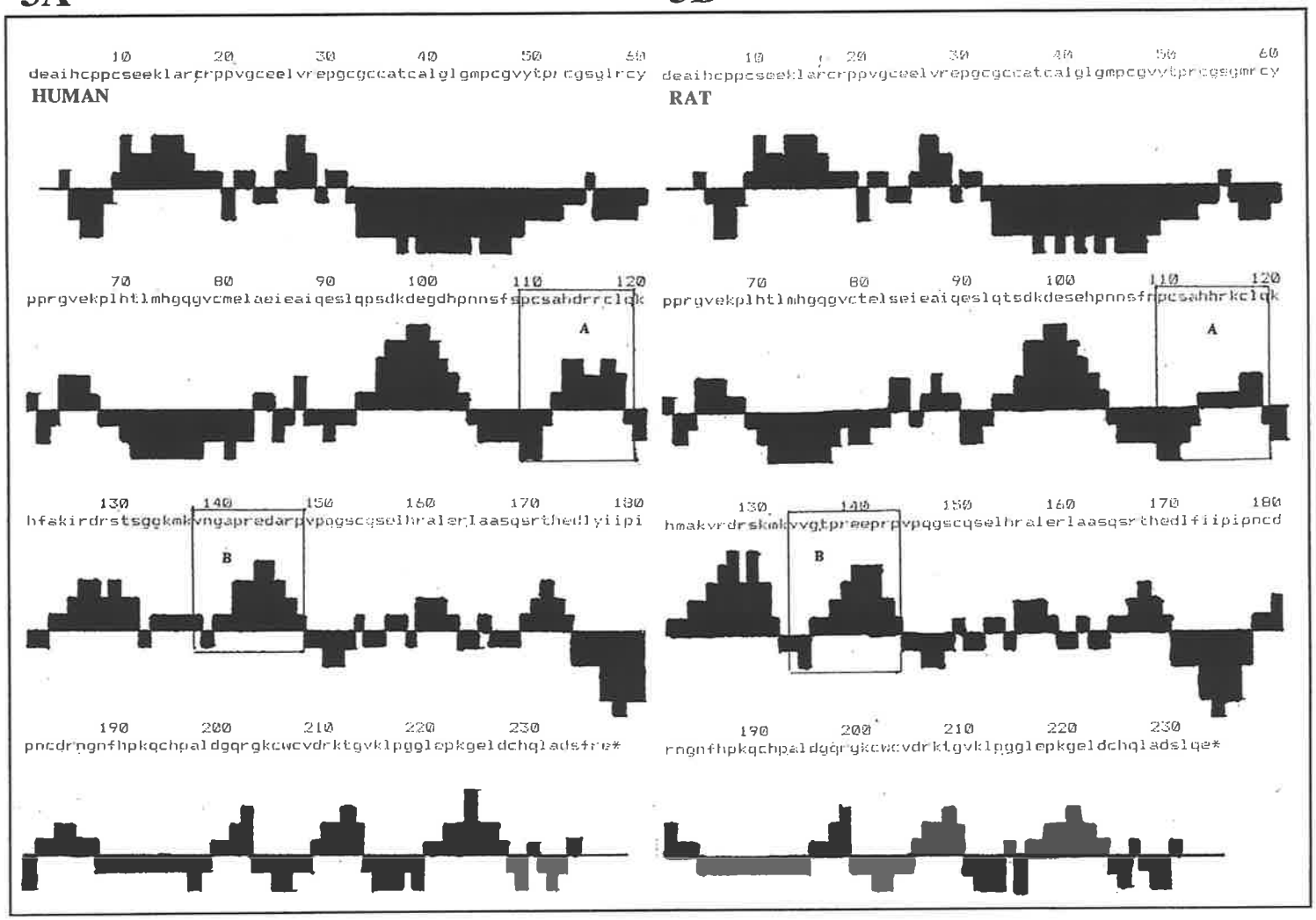
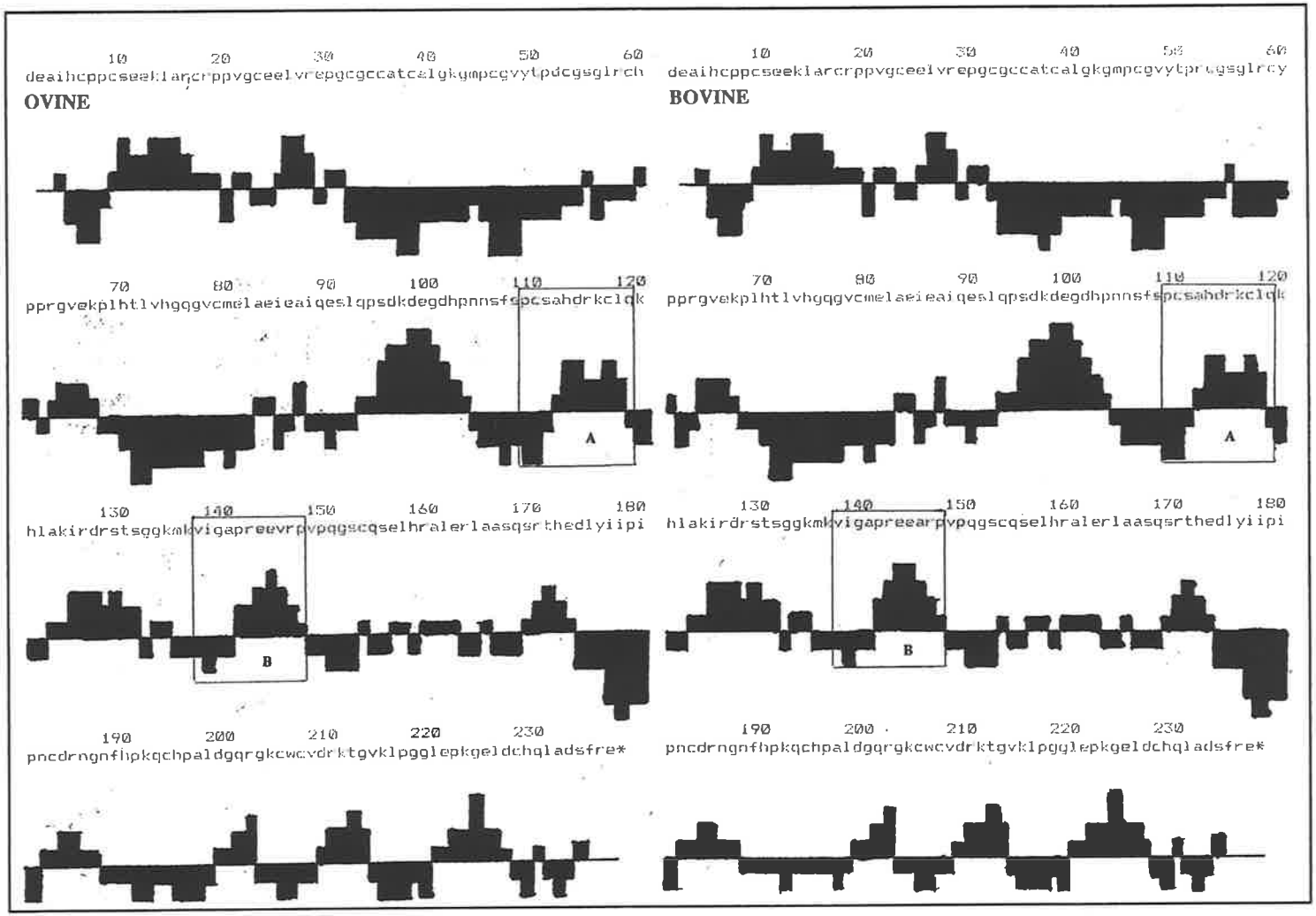
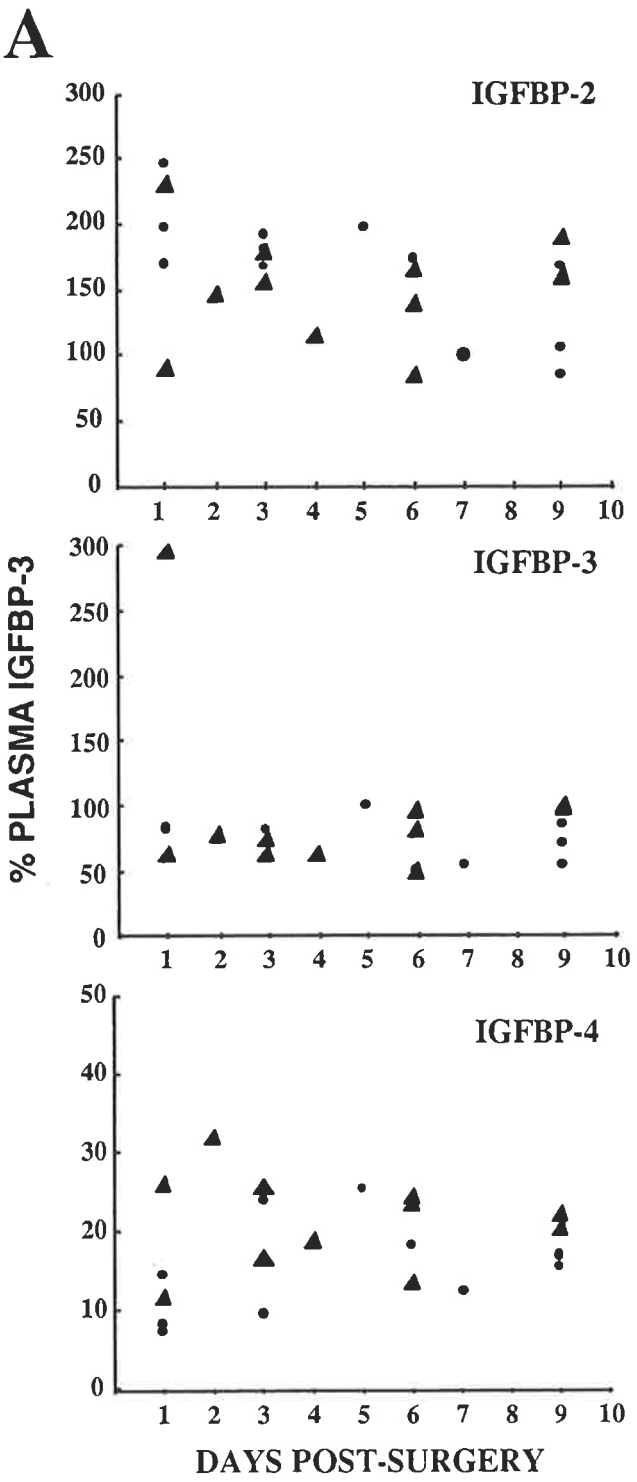


APPENDIX I (continued - Hopp and Woods, [1981] hydrophilicity)

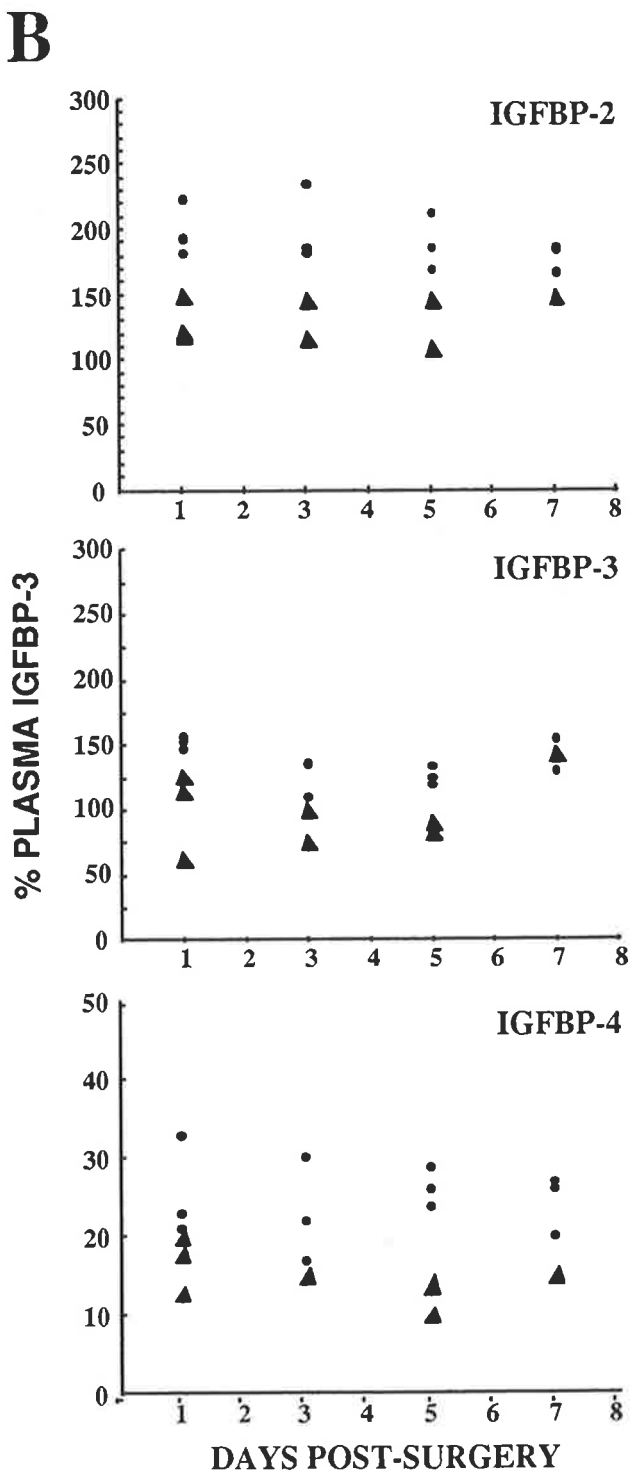
3C **3D**



APPENDIX I (continued- Hopp and Woods, [1981], hydrophilicity)



APPENDIX II. POST-SURGICAL STABILITY OF FETAL IGFBP LEVELS. Circulating IGFBP levels were assessed by Western ligand blotting from 1 to 10 days post-surgery, in both control (▲) and growth restricted (●) fetuses, operated on at 73 to 78 (A) or 107 to 109 (B) days gestaion. Autoradiographs were scanned and band intensities expressed as a percentage of a sheep plasma control electrophoresed on each gel. n = 3 for each group at each age.



APPENDIX II (continued, post-surgical stability of fetal IGFBP levels at 107 to 109 (B) days gestation). ▲ = normal fetuses. ● = growth restricted fetuses.