INSULIN-LIKE GROWTH FACTOR SYSTEM IN PREGNANCY

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A reverse transcription polymerase chain reaction (RT-PCR) digoxigenin enzyme linked immunosorbent assay (DIG ELISA) specific for guinea pig IGF-I mRNA was developed to quantify the expression of IGF-I. β-actin mRNA was also quantified by a similar procedure as internal reference. Relative abundance of IGF-I mRNA is expressed as the ratio of IGF-I to β-actin cDNA products of RT-PCR. Both IGF-I and β-actin mRNA DIG ELISAs were validated by comparison with their analysis by conventional nonisotopic PCR measured by densitometric analysis of ds cDNA of predicted size resolved by agarose gel electrophoresis and stained with ethidium bromide. The two methods compare favourably for both IGF-I and β-actin. IGF-I and β-actin mRNA were then routinely measured by PCR DIG-ELISA because of its superior throughput, sensitivity and reproducibility. IGF-I and IGF-II proteins in blood were measured by radioimmunoassay after size exclusion HPLC of plasma at pH 2.5. IGFBPs in plasma were measured by western ligand blot analysis.

The relative abundance of IGF-I mRNA in liver, was found to be increased by pregnancy in *ad libitum* fed guinea pigs. Pregnancy also increased plasma IGF-I concentrations in *ad libitum* fed guinea pigs. Reducing feed intake by 30% before and throughout pregnancy abolished this effect of pregnancy on both IGF-I mRNA in liver and IGF-I protein in plasma. Plasma IGF-II was unaffected by pregnancy but was reduced by undernutrition in both pregnant and nonpregnant animals. Plasma IGFBP-3 concentration was unaffected by pregnancy but was reduced by undernutrition regardless of pregnancy status. Pregnancy increased plasma IGFBP-1 levels in feed restricted but not in *ad libitum* fed animals. Undernutrition decreased plasma IGFBP-1 concentration in nonpregnant guinea pigs but increased it in pregnant animals. Pregnancy increased plasma IGFBP-2 concentration in nutritionally restricted animals only. Feed restriction increased concentrations of IGFBP-2 in plasma from pregnant animals only. Pregnancy increased plasma IGFBP-4 concentration in both *ad libitum* fed and nutritionally restricted animals. Nutrition had no effect on plasma IGFBP-4 levels.

Plasma IGF-I (y) was positively correlated ($r^2 = 0.68$, $p<0.002$)) to the relative abundance of hepatic IGF-I mRNA (x), $y = 105 + 65x - 1.4x^2$ when animals from all four treatments were combined. Because 68% of the variation in plasma IGF-I can be accounted for by variation in the relative abundance of IGF-I mRNA this strongly indicates that liver is the major source of endocrine IGF-I in guinea pigs. Overall therefore, approximately two-thirds of the IGF-I in blood of guinea pigs appears to come from liver.

Pregnancy increased hepatic IGF-I mRNA abundance 6-fold but increased plasma IGF-I concentration only 3-fold in *ad libitum* fed guinea pigs. This suggests that pregnancy increased both IGF-I synthesis and clearance of endocrine IGF-I. An alternate explanation could be that translation of IGF-I mRNA is saturable resulting in accumulation of untranslated IGF-I mRNA in liver at high levels of IGF-I expression.

In pregnant animals the relative abundance of hepatic IGF-I mRNA was positively correlated with maternal body weight ($r^2 = 0.61$, $p = 0.01$), weight gain during pregnancy ($r^2 = 0.69$, $p<0.01$) and uterine weight ($r^2 = 0.62$, $p = 0.01$), consisting of uterus, fetuses and placentae. Maternal hepatic IGF-I mRNA abundance was also positively correlated with fetal weight ($r^2 = 0.64$, $p<0.01$) and placental weight ($r^2 = 0.55$, $p = 0.03$). These positive associations indicate that IGF-I originating from maternal liver has endocrine actions on the mother, placenta and fetus.

Covariate analyses revealed that the number of fetuses per dam ($r^2 = 0.4$, $p<0.02$), total fetal weight ($r^2 = 0.72$, $p<0.001$) and total placental weight ($r^2 = 0.63$, $p<0.001$) were dependent on maternal plasma IGF-I concentrations but not any other measurements of the maternal IGF axis. Covariate analyses that included interactions between measurements of IGF endocrine components revealed that the ratio of the concentrations of IGF-I to those of IGFBP-2 in plasma of pregnant animals was a stronger positive determinant of uterine weight ($r^2 = 0.52$, $p<0.01$), fetal weight ($r^2 = 0.52$, $p<0.01$) and placental weight ($r^2 = 0.52$, $p<0.01$) than for any other measure of pregnancy phenotype. These associations suggest that IGFBP-2 may inhibit the growth promoting endocrine actions of IGF-I on the mother and conceptus.

Comparison between the concentrations of individual components of the IGF system and the ratio of the concentrations of IGFs/IGFBPs identified independent covariates of specific tissue weights. In pregnant guinea pigs the ratio [IGF-I]/[IGFBP-2] was positively correlated with weights of some maternal tissues as well as those of the fetuses and placentae. The same ratio [IGF-I]/[IGFBP-2] was found to be a correlate of weight of the gastrointestinal tract but this was negative. This suggests molecular complexes formed between IGF-I and IGFBP-2 has different biological roles at different tissue sites. The ratio [IGF-I]/[IGFBP-1] was also correlated with weights of tissues in pregnant guinea pigs but these are different from those found with [IGF-I]/[IGFBP-2]. All of these ratios that correlate with weights of maternal tissues, fetuses, placentae contain [IGF-I] and none contain [IGF-II]. This suggests pregnancy shifts somatic growth of the mother and conceptus from IGF-II dependence to IGF-I dependence. All of the associations between concentrations of the ratios of

concentrations of IGF endocrine and tissue weights in pregnancy appear to be IGF-I dependent. However, IGFBP-3 was identified as an independent covariate of selected tissues in the pregnant guinea pig suggesting IGFBP-3 may also have some IGF-independent actions. In the nonpregnant animals IGFBP-3 concentration is a correlate of the weights of different tissues to that seen in pregnancy suggesting pregnancy factors may differentially regulate the site of action, synthesis and/or turnover of IGFBP-3. In summary, pregnancy shifts the dependence of somatic growth on the IGF endocrine axis from IGF-II to IGF-I dependence.

In the nonpregnant guinea pig all ratios between concentrations of IGFs and IGFBPs that were correlated with tissue or organ weights contained [IGF-II]. The ratio [IGF-II]/[IGFBP-2] was found to be positively correlated with weights of almost all tissues studied. Therefore in this species IGF-II is the major IGF endocrine determinant of somatic growth unlike the situation in nonpregnant rats where IGF-I is the main endocrine IGF correlate of somatic.

In conclusion, liver has been identified as the major source of endocrine IGF-I in pregnancy in guinea pigs. Pregnancy increases hepatic abundance of IGF-I mRNA and circulating levels of IGF-I protein. These effects are dependent on maternal nutrition. Associations between maternal hepatic IGF-I mRNA abundance, plasma IGF-I concentration and pregnancy phenotype indicate that maternal endocrine IGF-I of hepatic origin is important in regulating maternal, fetal and placental growth in late pregnancy.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in text. I give my consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

Signed

Date 28th June 2002

Patricia Grant

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ABBREVIATIONS

A	adenine
ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] diammonium
AMP	adenosine monophosphate
AP	adaptor protein
bp	basepairs
C	cytosine
cDNA	single stranded DNA sequence complementary to RNA
C/EBP	CAAT-box/enhancer binding protein
CSF	cerebral spinal fluid
CV	coefficient of variation
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
E	efficiency of amplification
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	g force
G	guanine
GHR	growth hormone receptor
gpβ-actin	guinea pig β-actin
gpIGF-I	guinea pig IGF-I
hrs	hours
HNF	hepatocyte nuclear factor
IGFBP 1-6	insulin-like growth factor binding proteins 1-6
IGFBPrp	insulin-like growth factor binding protein related proteins
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
kb	kilobases
kDa	kilodaltons
l	litre
M	molar
MAP	mitogen-activated protein

MEK	MAP kinase kinase
mg	milligram
mins	minutes
ml	millilitre
mRNA	messenger ribonucleic acid
^{o}C	degrees Celsius
PAGE	polyacylamide gel electrophoresis
pM	picomolar
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium docedyl sulfate
SEM	standard error of the mean
T	thymine
T_m	melting temperature of DNA
UV	ultraviolet
V	volts
v/v	volume/volume
-ve	negative
w/v	weight/volume
µg	micro-gram
µl	microlitre
µM	micromolar

PUBLICATIONS ARISING FROM THE RESEARCH IN THIS THESIS

Abstracts

Pat Grant, Claire Roberts, Julie Owens and Phil Owens. (2000) Pregnancy changes the relationship between plasma concentrations of IGF-I and hepatic abundance of IGF-I mRNA. ICE 2000 Satellite Symposium. " Endocrinology and Development" P6.

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CHAPTER 1

INTRODUCTION

Introduction

Fetal growth and development and hence phenotype at birth are determined by interactions between the fetal genome, the placenta, the intrauterine environment, maternal adaptation to pregnancy, maternal factors including size, age and nutritional status before and during pregnancy and the transfer by the placenta of oxygen and nutrients to the growing conceptus. The potential growth trajectory of the fetus is determined by its genome, but other factors interact with the fetal genome to produce the actual growth trajectory. For example, the size of the fetus is heavily dependent on the delivery of glucose and oxygen to the fetus. Maternal undernutrition and/or impaired placental function reduce the delivery to the fetus of essential substrates resulting in intrauterine growth retardation (IUGR). IUGR is associated with increased rates of mortality and morbidity for the fetus as well as the newborn infant. It has also been shown that babies who are small at birth have increased risk of adult diseases such as diabetes, hypertension and heart disease (Barker, 1994). This has been suggested to be a result of permanent intrauterine programming. During embryonic and fetal life cells in different organs and systems replicate and differentiate at different stages of development. It is proposed that during these processes cells are most sensitive to permanent programming of patterns of organ structure, physiology and metabolism (Barker, 1994). These can predispose the individual to impaired function in adult life resulting in an increased risk of diseases. This is known as the fetal origins of adult diseases hypothesis, also known as the "Barker hypothesis'.

The placenta is the organ that delivers essential substrates to the fetus and provides signalling pathways between the mother and fetus. Weights of the human placenta and baby at birth have been shown to be positively correlated with the concentrations of insulin-like growth factor-I (IGF-I) in maternal plasma (Caufriez et al., 1994;McIntyre et al., 2000). The placental trophoblast is important in controlling maternal metabolism (Evain Brion, 1994). The

placental syncytiotrophoblast synthesises steroids, peptides, polypeptide hormones and cytokines that are normally produced by, or under the control of, the hypothalamus and pituitary in non-pregnant individuals (Petraglia et al., 1996). Mechanisms that regulate the partitioning of nutrients between maternal tissues and the conceptus are poorly understood. However, peptides such as placental lactogen and placental growth hormone variant, which possess a high degree of amino acid sequence homology with pituitary growth hormone, impart many of their effects through the insulin-like growth factor system (Talamantes, 1988). The *in vitro* cellular actions and *in vivo* pharmacological actions of IGFs include stimulation of cellular proliferation and differentiation, increasing uptake of amino acids, glucose uptake, inhibition of lipolysis and increasing synthesis of DNA, RNA and protein.

1.1 Physiological response to pregnancy

In many species pregnancy involves major anatomical, physiological and metabolic changes essential for both maternal and conceptus survival. Maternal adaptation to pregnancy involves increased adipose deposition early in pregnancy to ensure adequate energy reserve for successful lactation as well as enlargement of breast tissue for milk production. The maternal cardiovascular system expands and organs including the thyroid, kidney, liver, pancreas and pituitary increase in size (Metcalfe, 1988). Increased energy requirements of maternal tissues as well as those of the conceptus, result in the need for appropriate partitioning of nutrients to ensure fetal survival. Mechanisms regulating maternal or fetal supply of glucose, amino acids and/or free fatty acids appear to be controlled by the placenta. Many of the physiological changes that occur in the mother are responses to placental production of steroids, peptides, polypeptide hormones and cytokines (Petraglia et al., 1996;Robertson et al., 1994).

1.1.1 Regulation of substrate partitioning between the mother, placenta and fetus.

Endocrine alterations

The placenta synthesises many hormones including progesterone, estrogen, placental lactogen (PL), placental growth hormone variant, human chorionic gonadotrophin, leptin, adrenocorticotrophin (ACTH), thyrotrophin-releasing hormone, luteinizing hormone-releasing hormone, corticotropin-releasing hormone (CRH) and somatostatin (Petraglia et al., 1996;Stables, 1999;Yen, 1991). Some of these have direct actions on cells and others act indirectly through mediators such as insulin, glucocorticoids and IGFs (Evain Brion, 1994;Owens, 1991).

Pregnancy in the human is characterised by elevated blood concentrations of insulin, insulin resistance in peripheral tissues (Butte, 2000), high fasting glucose and amino acid levels in blood. Progesterone and estrogen have been demonstrated to induce insulin secretion by hypertrophy of the pancreatic islets of Langerhans in humans and animals (Cunningham, 1993). However, these hormones have different effects on maternal glucose status. Estradiol treatment significantly lowers glucose levels, whereas progesterone decreases tissue sensitivity to insulin and does not alter glucose levels (Yen, 1991).

Cortisol, PL and placental growth hormone antagonise the peripheral actions of insulin which promotes lipolysis, thereby increasing free fatty acid availability and increases protein breakdown in muscles consequently increasing amino-acid availability, thus providing alternate substrates to glucose for fetal and maternal tissues (Yen, 1991). Corticoid stimulation of lipolysis occurs only when insulin is insufficient to promote lipogenesis. Although cortisol binding globulin (CBG) transcortin increases with pregnancy there is still a

Placental hormones and growth factors in pregnancy

The placenta produces many hormones, steroids and cytokines that are either secreted by or regulated by the maternal hypothalamus and pituitary in non-pregnancy. Growth hormone for example, which is normally secreted by the pituitary into systemic blood in a pulsatile manner, is replaced by other somatogenic hormones including placental growth hormone that is secreted continuously into the maternal circulation in increasing amounts as pregnancy progresses (Caufriez et al., 1990;Evain-Brion, 1999). In addition the placenta produces PL and prolactin, both of which have a high degree of amino acid sequence similarity with pituitary derived growth hormone (Handwerger and Freemark, 2000) and it is suggested that these hormones, like GH, impart their actions directly and indirectly through the IGF system. Human and rhesus monkey placental syncytiotrophoblasts synthesize placental growth hormone, which has a high affinity for hepatic membrane GH receptors (Evain Brion, 1994). Maternal plasma levels of placental growth hormone correlate significantly with those of IGF-I in human pregnancy. In mothers with growth restricted fetuses low blood levels of both IGF-I and placental growth hormone are found at 28 and 36 weeks of pregnancy (McIntyre et al., 2000). Maternal levels of free GH, total GH and IGF-I in blood are positively correlated with birth weight (McIntyre et al., 2000) and maternal glycemia. Pregnant mothers with noninsulin-dependent diabetes mellitus (NIDDM) have elevated plasma levels of GH-binding protein (GHBP) whereas in those with insulin-dependent diabetes mellitus (IDDM), GHBP was decreased at both 28 and 36 weeks of gestation while free placental GH was increased at 36 weeks.

Maternal plasma concentrations of IGF-I are not related to plasma levels of PL (Caufriez et al., 1994;Evain-Brion, 1999). However, Iwashita *et al* (1992) observed a positive correlation between maternal plasma IGF-I and hPL levels. They also found that levels of PL in cord

blood at birth positively correlated with fetal blood levels of IGF-I and IGF-II at 33 weeks of gestation (Lassarre et al., 1991). These authors proposed that PL regulates fetal IGFs through fetal PL receptors prior to the expression of GH receptors, which first occurs postnatally.

Identification of the major site of synthesis and the mechanism that increases plasma IGF-I in pregnancy would allow greater understanding of the maternal adaptive mechanisms that sustain fetal growth. This may identify therapeutics for the prevention and or treatment of IUGR by manipulating the maternal IGF axis to alter maternal metabolism and partitioning of nutrients thus ultimately promoting fetal growth.

1.2 Insulin-like growth factors

Identification of growth factors

In the early 1950's *in vitro* actions of serum were identified as three separate biological activities, sulfation factor activity (SFA) related to longitudinal bone growth (Salmon and Daughaday, 1957), non-suppressible insulin-like activity (NSILA) which has the same biological actions as insulin, but cannot be suppressed by insulin antibodies (Froesch et al., 1966), and multiplication-stimulating activity (MSA) which is the component of serum that promotes cell proliferation (Dulak and Temin, 1973).

By the late 1940's and early 1950's it was known that GH from the pituitary gland promoted the longitudinal growth of bones and that this was associated with synthesis of chondroitin sulphate by chrondrocytes. In 1957 Salmon and Daughaday discovered that stimulation of the uptake of sulfate into cartilage *in vitro* was only achieved in the presence of normal rat serum and not with serum from hypophysectomized (pituitary deficient) rats. The addition of growth hormone to media containing cartilage and hypophysectomized rat serum could not stimulate

sulfate uptake. However, serum from hypophysectomized rats who had been treated with GH restored this effect. They proposed that GH stimulated the secretion into blood of a factor that promoted chondroitin-sulphate synthesis by chrondrocytes. This has been referred to as the somatomedin hypothesis. The bone growth promoting mediators of GH (also known as somatotropin) were collectively referred to as somatomedins (Daughaday, 1999).

In 1978 Rinderknecht and Humbel purified and chemically characterised NSILA from human plasma, which consisted of two similar biologically active forms, which they named IGF-I and IGF-II because of their structural similarity to pro-insulin and their mitogenic effects on cultured mammalian cells (Daughaday and Rotwein, 1989;Rotwein, 1991;Sara and Hall, 1990). Subsequent assessment of their biological activities revealed GH promoted production of IGF-I. The somatomedins were then renamed IGFs. IGF-I was identical to somatomedin C, while MSA isolated from conditioned medium of cultured rat liver cells was identified to be rat IGF-II (Dulak and Temin, 1973).

The human IGF-I cDNA sequence appeared 5 years later (Jansen et al., 1983). Organisation and structure of the IGF-I gene has been published for many species including human (de Pagter Holthuizen et al., 1986;Rotwein et al., 1986), rat (Shimatsu and Rotwein, 1987), mouse (Bell et al., 1986), chicken (Kajimoto and Rotwein, 1991) and guinea pig (Bell et al., 1990).

1.2.1 Insulin-like growth factor-I

The insulin family includes insulin, relaxin, nerve growth factor, IGF-I and IGF-II. Human IGF-I is a basic 70 amino acid polypeptide with a molecular weight of 7,646 daltons and IGF-II is a slightly acidic 67 amino acid polypeptide having a molecular weight of 7,471 daltons. IGF-I and IGF-II are single chain polypeptides having 70% homology with each other and

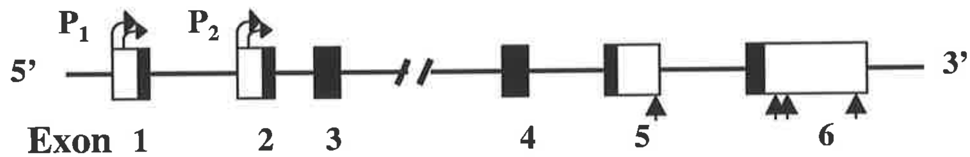
approximately 50% homology with proinsulin (Froesch and Zapf, 1985;Rinderknecht and Humbel, 1978). Both IGFs have A and B domains (named after the A and B chains of insulin) joined by three disulphide bridges similar to proinsulin. However, IGFs and pro-insulin have unique connecting C-peptides (Froesch and Zapf, 1985). The IGFs have a short D extension peptide at the carboxy-terminus of the A domain which is not present in either pro-insulin or insulin (Sara and Hall, 1990).

1.2.1.1 IGF-I gene

The human IGF-I gene is mapped to the long arm of chromosome 12 (12q22-q24.1). The IGF-I gene extends over 50 kb (chicken) to 80 kb (rat) of chromosomal DNA. The structure of the human and rat (Hoyt et al., 1992) IGF-I gene consists of 6 exons and 5 introns (Figure 1.1). Exons 1 and 2 contain 5' untranslated sequences and the NH_2-terminus of the signal peptide, exons 3 and 4 encode the remainder of the signal peptide and the mature IGF-I polypeptide, while exons 5 and 6 encode for COOH-terminal regions of the alternative E domains of the prohormone (Rotwein, 1991).

Multiple forms of IGF-I mRNA transcripts occur as a result of several mechanisms (Figure 1.1). These include usage of different promoters and transcription start sites leading to distinct 5' untranslated regions and different NH_2-terminal coding domains, alternate splicing of exon 5 and 6 for the production of transcripts that encode peptides with distinct carboxyl-termini, known as IGF-Ia and IGF-Ib prohormones, differences in the 3' untranslated region and variable polyadenylation.

IGF-I Gene



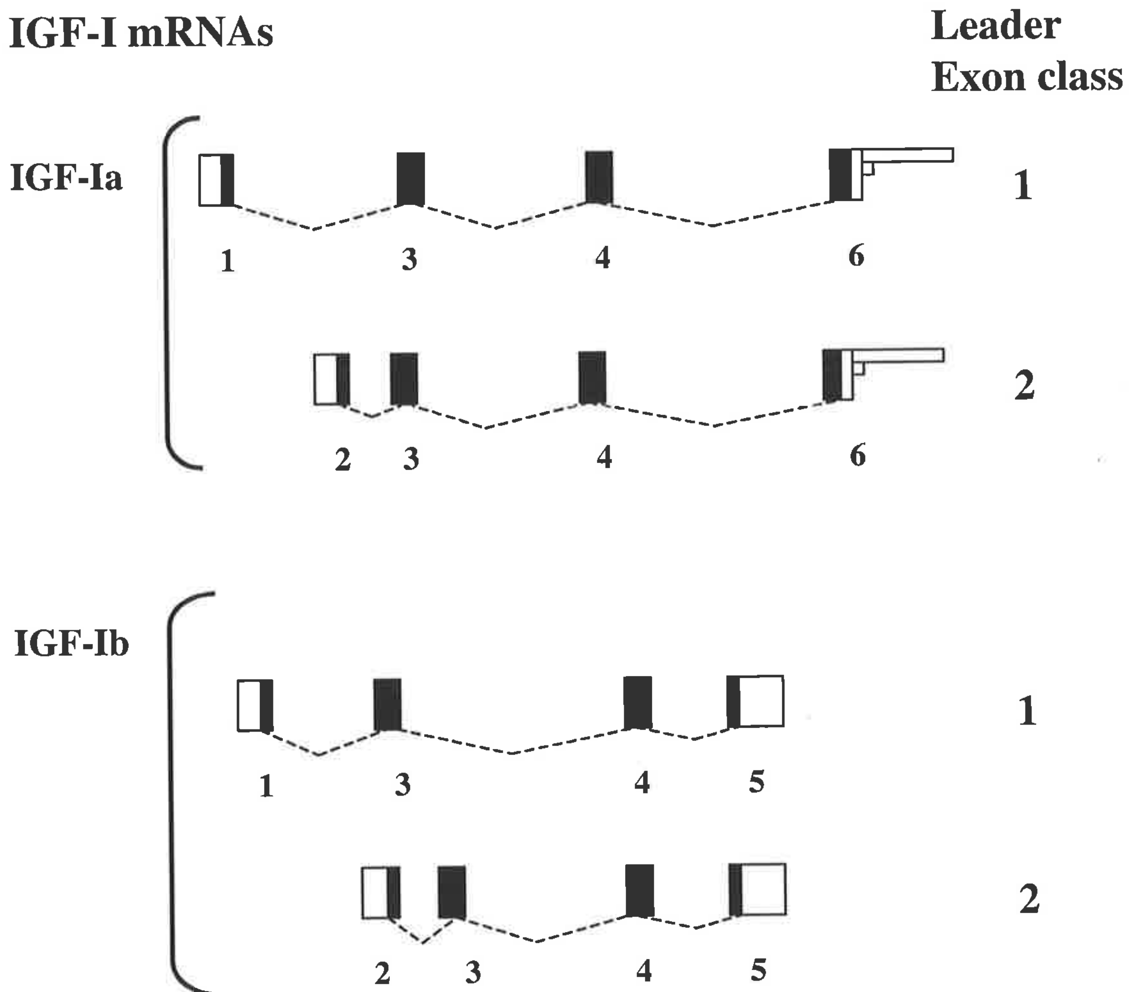


Figure 1.1 Structure of the human IGF-I gene and the different IGF-I mRNAs

Boxes represent exons and the coding regions are black and the non-coding are white. Solid back lines represent introns. Polyadenylation sites are indicated by arrows, the promoter regions by the letter P, and bent arrows indicate transcription initiation sites. Dashed lines indicate alternate splicing of exons 5 and 6 producing alternate carboxy-terminal Ea and Eb domains and alternate splicing of exon 1 and 2 producing different leader peptides. IGF-I mRNAs transcribed from exon 1 and exon 2 are also known as class 1 and class 2 transcripts respectively. (adapted from Rotwein 1991, 1999)

gene also contains a cAMP responsive element. Initiation of IGF-I transcription by cyclic AMP has been shown to involve protein kinase A (PKA) (Thomas et al., 1996). Parathyroid hormone, parathyroid hormone-related hormone and prostaglandin E_2 induce IGF-I expression by this pathway whereas 17β-estradiol suppresses c-AMP induced transcription of IGF-I. Glucocorticoids, such as cortisol, decrease transcription of P1 but the exact responsive elements have not been identified. Growth hormone binding to the growth hormone receptor activates a cascade of transduction pathways, but the exact elements responsible for IGF-I induction have not been unravelled. (Holthuizen, 1999).

Human cell lines SK-N-MC (human neuroepithelioma) and OVCAR-3 (human ovarian carcinoma) expressing IGF-I mRNA have been shown to have different levels of activity at promoters P1 and P2. In OVCAR cells P2 is four times more active than P1. In SK-N-MC cells P1 is three times more active than P2. A recent study has identified two GATA elements, GATA-A and GATA-B, in promoter P1 of exon 1 of cultured SK-N-MC, OVCAR-3 and C6 cells (Wang and Adamo, 2000). Mutation of GATA-A resulted in stimulation of promoter P1 activity in SK-N-MC cells while mutation of GATA-B inhibited this activity. However, little or no effect was apparent on promoter activity in the other cell lines tested. This suggests that there is cell specific regulation of IGF-I gene promoter usage (Jansen et al., 1992). Examples above show that the synthesis of IGF-I mRNA is intricately managed by each cell type through interactions with both tissue specific and ubiquitously expressed transcription factors. These appear to act both independently and in combination.

Dietary protein restriction has been shown to affect the transcription of several genes by alteration of the abundance and activity of a number of liver transcription factors (Marten et al., 1996). Some of these include hepatocyte nuclear factors (HNF)-1, -3 and –4 as well as the CCAAT/enhancer-binding protein (C/EBP) family. The ubiquitous transcription factor Sp1

increased DNA binding activity, whereas nuclear factor 1 (NF1) DNA binding activity was unaltered by dietary protein deprivation (Marten et al., 1996). Smaller NF1-like proteins were decreased by protein deprivation and their DNA binding activity reduced. IGF-I regulatory binding sites for liver transcription factors have been identified in the promoter region of exon 1 and nutritional protein restriction alters the abundance and activity of liver transcription factors HNF-1, C/EBP, Sp1 and NF1. This suggests this may be a mechanism through which protein restriction regulates IGF-I gene transcription. IGF-I gene expression in cultured ovine hepatocytes was increased by the synergistic action of GH and the concentration of amino acids in the cultured media (Wheelhouse et al., 1999). The expression of liver transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) isoforms, liver-enriched activating protein (LAP) and liver-enriched inhibitory protein (LIP) were differentially affected by dietary protein restriction (Wheelhouse et al., 1999). Dietary protein restriction decreased the ratio of LAP to LIP suggesting that more of the liver-enriched inhibitory protein binds the CCAAT/enhancer sequence in the IGF-I promoter thus inhibiting IGF-I gene expression. Insulin also has been shown to regulate IGF-I gene expression in cultured rat hepatocytes, H4IIE cells and CHO-IR cells by an insulin-responsive binding protein (IRBP) that interacts with an AT-rich site in the V region (footprinted sequence) in the IGF-I gene (Kaytor et al., 2001). The level of IRBP and interaction with the AT-rich site increases with concentrations of insulin. Therefore IGF-I gene expression is increased by insulin through insulin-response nuclear factors.

The nucleotide sequences of the multiple transcription initiation sites in the IGF-I gene are highly conserved between mammalian species (Hall et al., 1992;Jansen et al., 1991;Kajimoto and Rotwein, 1991;Ohlsen et al., 1993). Transcription initiation sites commonly require a GC-rich promoter that contains a TATA box. Mammalian IGF-I gene promoters do not contain TATA elements and are not GC rich. These promoters within the genes are not

constitutively active but are regulated by cellular differentiation and development (Smale and Baltimore, 1989).

1.2.1.2 IGF-I polypeptide

IGF-I peptide contains a NH_2-terminal B domain of 29 amino acids, a C domain of 12 residues an A domain of 21 amino acids and a COOH- terminal D domain of 8 residues. There is 77% amino acid sequence identity between mammalian and non-mammalian species. The main variations are found in the C domain with minor variations in the D domain. Amino acids in the A and B domain have been shown by mutagenesis to be important for binding to the IGF type-I receptor and to IGF binding proteins (IGFBPs) 1 and 3 (Rotwein, 1991). There are 3 amino acid differences between the rat and human, a proline for aspartic acid at position B20, an isoleucine for serine at position C35 and threonine for alanine at position D67. The mouse IGF-I amino acid sequence, identified from a cDNA mouse liver library (Bell et al., 1986), has one more substitution than the rat, an alanine for serine at position D69. The cow, pig and guinea pig mature IGF-I polypeptides are identical to human IGF-I (Bell et al., 1990;Daughaday and Rotwein, 1989). Jansen *et al* (1983) discovered that IGF-I is synthesised as precursor proteins with different C termini in the E domain. In addition two IGF-I cDNAs (IGF-IEa and IGF-IEb) have been identified in the human (Rotwein et al., 1986), (Conover et al., 1993), rat (Lowe et al., 1988) and mouse (Bell et al., 1986) derived by alternate promoter usage from a single gene.

Alternate splicing of the IGF-I transcripts also produces precursor proteins with different COOH-termini (E region). IGF-IEa in mammalian species has a common 16 amino acid region in the E domain with only one substitution and a fairly highly conserved Ea domain. However in non-mammalian vertebrates both Ea and Eb domains are more variable. The Eb

region in IGF-IEb is longer and has a greater degree of amino acid substitution between species than found in the Ea region of IGF-IEa (Rotwein, 1991) in both mammalian and non-mammalian species. A major difference between IGF-IEa and IGF-IEb in the rat is due to the insertion of 52 nucleotides in the cDNA encoding the E domain of the IGF-IEb prohormone, yet the length of IGF-IEb protein only differs from that of IGF-IEa by 6 amino acids. Alternate splicing of exon 4 (52 base insert) in the rat and mouse changes the amino acid reading frame of IGF-IEb introducing a termination codon in exon 5, which thus accounts for the small difference in length. Two alternate IGF-I mRNA forms are also produced in humans, but by a different mechanism from that which occurs in rats. In the human IGF-IEa and IGF-IEb mRNA are products of mutually exclusive splicing of either exon 4 or 5 to exon 3 (Foyt, 1991). In the human splicing of exon 3 to exon 4 encodes IGF-IEb precursor of 77 amino acids. Different mechanisms of alternate splicing between species produces diversity in the E domain of IGF-I.

Another difference between IGF-IEa and IGF-IEb mRNAs is the length of the 3' untranslated regions due to variable polyadenylation (Lund et al., 1989). Using rabbit reticulocyte lysate as an *in vitro* cell free translation system the half life of the large IGF-I transcript (7.0 - 7.5 kb) was shown to be much shorter than the smaller (0.9 - 1.2 kb) IGF-I mRNAs (Hepler et al., 1990). Zhang *et al* (1998) showed amino acid deprivation of cultured rat hepatocytes produced rapid degradation of the 7.5 kb, 1.6 kb and 0.7 - 1.0 kb IGF-I mRNA transcripts. This suggests that in tissues where the 7.0 - 7.5 kb species dominates IGF-I mRNA may be highly unstable, resulting in a decline in translatable message to encode for the mature protein. Two potential glycosylation sites have been identified in the E region of rat IGF-IEa prohormone. Insertion of the 52 bp exon 4 produces rat IGF-IEb with no glycosylation sites at all. The rabbit reticulocyte *in vitro* translation system was used to demonstrate that either one or both of the predicted glycosylation sites can be used to produce three glycosylation forms

of rat IGF-IEa prohormone. Three forms were identified and incubation with N-glycanase F converted these to a single form as seen by PAGE and autoradiography (Bach et al., 1990). Different glycosylated forms of IGF-Ia may differ in stability, bioactivity, secondary structure and intracellular protein transport. Both precursors appear to give rise to the same mature secreted IGF-I protein, however differences in biological function have been postulated for different forms of IGF-Ia and IGF-Ib.

1.2.2 Insulin-like growth factor-II

1.2.2.1 IGF-II gene

The human IGF-II gene is mapped to chromosome 11p15.5 and is 3' to the insulin gene and less than 200 kb 5' to the H19 gene (Rotwein, 1999). These linked genes are mapped to chromosome 7 in the mouse (Rotwein and Hall, 1990) and chromosome 1 in the rat (Rechler, 1991). The human IGF-II gene contains 10 exons and 9 introns. The polypeptide coding region extends over 3 exons (exon 8 to 10 in humans and exons 4 to 6 in rodents). Human exon 8 (rat exon 4) codes for the 24 amino acid signal peptide and the mature IGF-II peptide and human exon 9 (rat exon 5) encodes the first 11 residues of the E domain. The remaining E domain and the 3' untranslated region is encoded by human exon 10 (rat exon 6) (Rotwein, 1991;Rotwein, 1999). Multiple RNA transcripts are produced from the IGF-II gene. This is due in part to differential use of four promoters (P1, P2, P3, P4) in human IGF-II and three (P1, P2, P3) for rodent IGF-II. In addition to these variable transcription initiation sites, variable RNA polyadenylation occurs in exon 10 for human and exon 6 for rodents. These mechanisms interact to produce numerous transcripts whose physiological significance has yet to be determined. In humans IGF-II is expressed in many fetal and adult tissues whereas rat IGF-II is abundantly expressed in embryonic and fetal tissues, with no significant expression

in adults except for in the brain and spinal cord (Rechler, 1991). Expression from promoters P3 and P4 has been shown in fetal and non-hepatic adult tissues. In human adult liver P2, P3 and P4 are inactive. P1 directs initiation of transcription of IGF-II postnatally (van Dijk et al., 1991). In rats, IGF-II gene promoters P1, P2 and P3 are silenced in liver after weaning.

1.2.2.2 IGF-II polypeptide

IGF-II is a 67 amino acid single chain polypeptide similar to IGF-I and pro-insulin in structural organisation with an amino-terminal B domain of 28 amino acids, C domain of 12 amino acids, an A domain of 21 amino acids, followed by a 6 residue carboxy-terminal D region (NH2-B-C-A-D-COOH) (Daughaday and Rotwein, 1989;Rechler, 1991;Rotwein, 1999). The amino acid sequences of IGF-II from many mammalian and several non-mammalian vertebrates have been determined. Variant forms of IGF-II have also been identified in the human and rat and have altered affinity for the type-I IGF-I receptor compared with native IGF-II. However the significance of these variants has yet to be determined. Replacement of leucine for tyrosine 27 in the B domain reduces the affinity of IGF-II for the IGF type-I receptor. IGF-II is synthesised as a precursor protein with 88 or 89 amino acid carboxy-terminal E domain, which unlike IGF-I is invariant. Some human tumours secrete large forms of pro-and prepro- IGF-II possessing the E domain which produce hypoglycaemia (Rotwein, 1991;Rotwein, 1999).

1.3 Insulin-like growth factor receptors

The mitogenic actions of both IGF-I and IGF-II are mediated by the type I IGF receptor, insulin receptors and hybrid type I IGF /Insulin receptors. Type I IGF receptor is similar in structure and has a greater than 50% amino acid sequence homology with the insulin receptor.

Type I IGF receptor is encoded by a single gene spanning 100 kb of DNA. The type I IGF receptor is a heterodimer consisting of a pair of an alpha and beta subunits linked by disulphide bonds. The alpha subunit is extracellular and contains a cysteine rich domain that is responsible for the binding of IGF-I, IGF-II and insulin. The beta subunit is comprised of a hydrophobic transmembrane domain, a juxtamembrane domain, intracytoplasmic tyrosine kinase enzyme domain with an ATP binding site, and a carboxy-terminal domain. Typically type I IGF receptor binds to IGF-I with higher affinity than IGF-II and binds insulin with 500 to 1000 times lower affinity (De Meyts, 1994).

There is a fourth receptor which essentially only binds IGF-II and does not share any sequence homology with the type I IGF or insulin receptor (Kiess, 1999). The type II IGF receptor is a single chain glycoprotein comprised of 2,264 amino acids of extracellular domain, 23 amino acids of transmembrane domain and 164 amino acids of intracellular domain. It is also known as the cation-independent mannose-6-phosphate receptor (CIM6PR). The extracellular domain binds the ligands and the intracellular domain regulates movement within cellular compartments. It has no established catalytic activity in response to IGF-II binding. The CIM6PR clears IGF-II from cells and recycles lysosomal enzymes glycosylated with mannose-6-phosphate. IGF-II receptor knockout mice fail to clear circulating and tissue IGF-II resulting in the development of organ overgrowth due to mitogenic actions of IGF-II on type I IGF receptors (D'Ercole, 1999). Direct signalling of IGF-II through the CIM6PR has been observed (Nissley, 1999), however, the mechanism is unclear.

In many cell lines and tissues expressing the type I IGF and insulin receptors, hybrid receptors may form. The hybrid receptors bind IGF-I with similar affinity to that of the type I IGF receptor. The type I IGF /insulin hybrid receptor forms by the dimerization between type I IGF receptor alpha/beta subunit hetero-monomer and an insulin receptor alpha/beta subunit.

Type I IGF/insulin hybrid receptors were purified from human placenta (Kasuya et al., 1993) and their abundance is increased in placenta from hyperinsulinaemic women with gestational hypertension, compared with placentas from normal pregnant women with gestational hypertension (Valensise et al., 1996). In obese humans, plasma levels of IGF-I and insulin correlate respectively with the abundance of type I IGF receptor and insulin receptor in skeletal muscle, which then determines the abundance of hybrid receptors in this tissue (Federici et al., 1998). Circulating levels of insulin and IGF-I and ratio of the abundance of type I IGF receptor/insulin receptor has been proposed as a mechanism determining the abundance of hybrid receptors. It has been suggested that hybrid receptors provide an alternate signalling pathway for IGF-I but the relative numbers and ratio may be important in conveying unique biological functions.

1.3.1 Regulation of type-I IGF receptor abundance

IGF-I receptor abundance is developmentally regulated in most tissues. The abundance of the type I IGF receptor in rat liver progressively decreases, while expression of hepatic IGF-I mRNA and plasma IGF-I peptide increase with postnatal age. The type I IGF receptor appears as early as the eight-cell stage prior to implantation in the mouse embryo. IGF-I and insulin are not expressed until after implantation, however, IGF-II protein has been detected as early as the two-cell stage, suggesting IGF-II action may be mediated by the type I IGF receptor early in mouse embryonic development. Platelet-derived Growth Factor and basic Fibroblast Growth Factor increase the abundance of type I IGF receptor in cultured human fibroblasts (Werner, 1991). Treatment with estrogens or with follicle stimulating hormone increases the level of type I IGF receptor mRNA *in vivo* in ovaries and *in vitro* in cultured granulosa cells (Werner, 1991). Fasting increases the abundance of the type I IGF receptor by 1.6 to 2.5 fold in a number of rat tissues including lung, testes, kidney, stomach and heart (Lowe et al., 1989).

1.3.2 Type I IGF receptor Intracellular signalling

Binding of IGF-I to the cysteine rich domain of the extracellular α-subunit of the IGF type I receptor initiates a sequence of signalling events commencing with autophosphorylation of the receptor β-subunit. This permits the binding of other intracellular proteins, which are subsequently tyrosine phosphorylated. IGF-I can initiate at least two cascades of intracellular proteins one of which activates the MEK, MAP kinases that ultimately can initiate cellular proliferation. Secondly, IGF-I activation of the type I IGF receptor has been shown to stimulate the phosphatidylinositol 3-kinase (PI3-kinase) which leads to the promotion of glucose transport, carbohydrate metabolism and antiapoptosis (Kato et al., 1993;Kulik et al., 1997;Thomson et al., 1997).

1.4 Insulin-like growth factor binding proteins

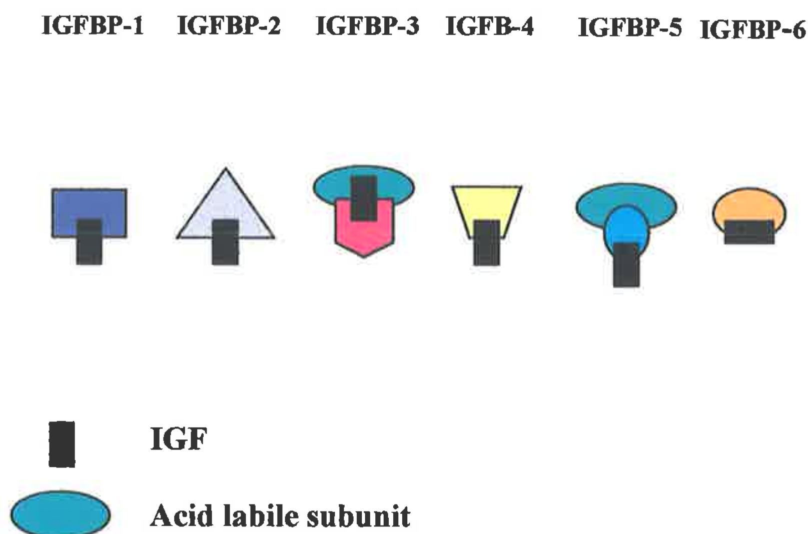
1.4.1 Classification

The IGFs circulate bound with high affinity to carrier proteins known as insulin-like growth factor binding proteins (IGFBPs), of which 6 have been identified. These are referred to as IGFBP-1 to –6 (Figure 1.2). This class of proteins has been recently enlarged to include IGFBP-related proteins-1 to -5 (IGFBP-rp) and collectively are referred to as the IGFBP superfamily (Hwa et al., 1999). IGFBP-1 to -6 share a structural similarity of a high number of cysteines (16-20), which are clustered at the amino-terminus and the carboxy-terminus. It has been proposed that these two terminal domains provide the tertiary site for high affinity binding of IGF-I and IGF-II. IGFBP-rp-1 to -5 and the IGFBPs, have identical amino-terminal

domains and different carboxy-terminal domains that explains their low affinity binding for IGFs (Hwa et al., 1999).

Figure 1.2 Circulating molecular complexes of IGFs to IGF-binding proteins

Adapted from (Wetterau et al., 1999).



1.4.1.1 Actions of insulin-like growth factor binding proteins

IGFBPs act to transport the IGFs to tissues, modulate their bioavailability and bioactivity, control clearance of IGF-I and IGF-II and regulate the amount of each IGF presented to their receptors. IGF independent actions of IGFBPs have also been reported (Baxter, 2000;Wetterau et al., 1999). IGFBPs are expressed by many cell types and occur in most biological fluids. IGFBP-3 is the major binding protein in blood that circulates as a 150 kDa complex consisting of either IGF-I or IGF-II with IGFBP-3 and an acid labile subunit (ALS). This 150 kDa complex accounts for ~75% of the IGF-I and II in blood in adult humans. The remainder of the IGFs are complexed to the other binding proteins IGFBP-1, 2, 4, 5, and 6.

The fraction of free IGF-I and IGF-II reported varies considerably, ranging from undetectable to 19% for IGF-I and 1% for IGF-II (Frystyk et al., 1994).

Human IGFBP-1 has a molecular weight of 25.3 kDa and the cDNA encodes for a 259 amino acid precursor with a 25 amino acid prepeptide and a 234 amino acid mature protein with an arginine-glycine-aspartic acid (RGD) sequence near the end of the carboxy terminus. This sequence binds to cell surface integrin receptors. IGFBP-1 binds to the $\alpha_5\beta_1$ integrin in porcine aortic cells (Jones et al., 1995). On a nonreducing SDS-PAGE gel human IGFBP-1 migrates between 25 and 31 kDa (Ooi and Boisclair, 1999). IGFBP-1 has equal affinity for IGF-I and II and is posttranslationally phosphorylated, altering its charge and causing a conformational change, which increases its affinity for IGF-I. Phosphorylation of IGFBP-1 does not affect IGF-II affinity. IGFBP-1 associated with integrin receptors indicates IGFBP-1 may deliver IGF-I to specific target cells. Phosphorylation also appears to protect IGFBP-1 from proteolysis. In pregnancy nonphosphorylated forms are susceptible to protease actions, which lower IGFBP-1 affinity for IGF-I and thereby increase IGF bioavailability (Clemmons, 1991;Gibson et al., 2001;Westwood et al., 1997).

IGFBP-1 is developmentally regulated in fetal rats. IGFBP-1 mRNA has been identified in the liver of fetal rats in mid gestation, while its expression appeared in nonhepatic tissues (stomach, heart, lung and kidneys) at lower levels late in gestation. The human fetal liver also expresses IGFBP-1 (Ooi and Boisclair, 1999). In human adults the liver and decidua from late pregnancy are major sites of IGFBP-1 synthesis. The protein is found in serum, lymph, CSF and in very large amounts in amniotic fluid. IGFBP-1 was initially purified from human amniotic fluid and/or human decidua. Serum IGFBP-1 levels are regulated by insulin and corticosteroids. Feeding reduces plasma IGFBP-1 and fasting increases circulating levels. IGFBP-1 is a glucoregulator (Baxter, 1995). Insulin suppresses serum IGFBP-1 through a

PEPCK-like response sequence in the promoter region of IGFBP-1 gene (O'Brien, 2001). IGF-I stimulated glucose uptake is inhibited by IGFBP-1 *in vivo*. IGFBP-1 is elevated in a number of catabolic disorders including malnutrition, poorly controlled diabetes, and intrauterine growth retardation (Collett Solberg and Cohen, 1996).

Rat IGFBP-2 was originally purified from BRL-3A cell conditioned media and is a 32 kDa protein with a RGD sequence at the C-terminal end similar to IGFBP-1. Binding to the integrin receptor $\alpha_5\beta_1$ has been demonstrated using Ewing sarcoma cells (Strain A673) only when IGFBP-2 is bound to IGF-I or IGF-II (Rauschnabel et al., 1999). IGFBP-2 binds heparin and heparin sulphate that are components of cell surface proteoglycans and the extracellular matrix. Bovine IGFBP-2 was purified from the MDBK cell line and revealed 87% amino acid sequence homology with the rat IGFBP-2. The IGFBP-2 protein migrates at 34 kDa on a non-reducing SDS-PAGE gel. IGFBP-2 is not glycosylated or phosphorylated, but fragments have been identified in serum of newborn fasted pigs and in follicular fluid suggestive of protease activity. In the fetal rat IGFBP-2 is expressed in high levels during early embryonic life and expression declines after birth. Circulating levels of IGFBP-2 increase with prolonged fasting, protein restriction, hypophysectomy (rat), GH deficiency (human), diabetes and intravenous infusion of IGF-I (Clemmons, 1991;Thissen et al., 1994).

IGFBP-3 protein was originally purified from human plasma, and is 264 amino acids long. The protein is N-glycosylated and circulates in serum in the glycosylated form with a molecular weight of 40-45 kDa as determined by nonreducing SDS-PAGE. Biological activity of IGFBP-3 is unaffected by the degree of glycosylation but susceptibility to proteolysis may be altered. Phosphorylated IGFBP-3 is secreted from human skin fibroblasts (Coverley and Baxter, 1995). Phosphorylation of IGFBP-3 by casein kinase 2 does not alter the binding affinity for either IGF-I or II but protects IGFBP-3 from degradation by plasmin

and cysteine proteases. Phosphorylated IGFBP-3 has reduced affinity for the ALS complex and cell surfaces (Coverley et al., 2000). IGFBP-3 contains a cell-surface recognition site (heparin-binding domain) which allows IGF-independent binding to bovine endothelial cells and other studies have proposed an IGFBP-3 receptor in breast cancer cells. Further evidence suggests that type V receptor for transforming growth factor-β (TGF-β) may be an IGFBP-3 receptor in mink lung cells (Baxter, 2000;Booth et al., 2002). Serum levels of IGFBP-3 are age dependent, increasing from birth, peaking at puberty and declining thereafter in the human.

IGFBP-3 is expressed in mouse embryonic tissue at day 18 but is detected slightly earlier in the rat embryo at day 14. In the adult rat the liver, kidney, uterus, placenta, ovary, stomach and heart abundantly express IGFBP-3. Circulating levels of IGFBP-3 are increased by growth hormone, IGF-I, adolescence, acromegaly and are reduced by undernutrition, diabetes, dexamethasone and hypopituitarism (Clemmons, 1991). IGFBP-3 both inhibits and potentiates IGF actions. IGFBP-3 inhibited IGF-I stimulated proliferation of immortalized human chondrocytes (Matsumoto et al., 2000). IGFBP-1 (10 μg/l to 250 μg/l) inhibited IGF-I (0.1 μg/l) and -II (1 μg/l) but not insulin (5 μg/l) stimulated glucose consumption in mouse Balb/c3T3 fibroblasts. IGFBP-1 at concentrations of 1 μg/l to 50 μg/l inhibited IGF-I to a greater extent than IGF-II. In comparison IGFBP-3 at 290 μg/l inhibited 80% and 60% of IGF-I and IGF-II stimulated glucose consumption in mouse Balb/c3T3 fibroblasts respectively although IGFBP-3 had a greater inhibitory effect on IGF-II than IGF-I (Okajima et al., 1993). Preincubation of BALB/c3T3 fibroblasts with IGFBP-3 followed by coincubation with IGF-I and IGFBP-3 potentiated this inhibitory effect 50-fold. However other studies have reported IGFBP-3 stimulation of IGF-I mitogenic activity (Cortizo and Gagliardino, 1995). This suggests that IGFBP-3 has multiple actions that are tissue and cell dependent.

The existence of proteases for IGFBPs (see also 1.4.1.2) was first identified when discrepancies between the estimation of IGFBP-3 in pregnant serum by western ligand blots and radioimmunoassays were reported (Davenport et al., 1990;Gargosky et al., 1990b). Other studies reported IGFBP-3 to be proteolytically modified in severe illness, malnutrition, malignancy, diabetes, and in the maternal serum of complicated pregnancies. Many studies have examined the effect of IGFBP-3 proteolysis in pregnancy showing that fragments have a reduced affinity for iodo-IGF-I and iodo-IGF-II which suggests greater availability of IGFs to target tissues (Suikkari and Baxter, 1992). IGFBP-3 has also been suggested to inhibit IGFBP-4 proteolysis. Conditioned media of human dermal fibroblasts cultured with increasing concentrations of IGF-I (5, 50 and 100 ng/ml) resulted in proteolysis of IGFBP-4. The addition of excess IGFBP-3 was able to block IGF induced proteolysis of IGFBP-4 (Donnelly and Holly, 1996). The mechanism underlying the inhibition of IGFBP-4 proteolysis possibly involves the heparin binding motif sequences that are located within the thyroglobulin type-1 repeat motif found in IGFBP-3, IGFBP-5 and IGFBP–6. IGFBP-3, -5 and –6 were demonstrated to inhibit IGFBP-4 degradation of MC3T3-E1 murine osteoblasts conditioned media. Synthetic C-terminal IGFBP peptides (IGFBP-3, IGFBP-5 and IGFBP-6) containing the thyroglobulin type-1 motif inhibited IGFBP-4 proteolysis in a dose dependent manner (Fowlkes et al., 1997). These studies suggest that the roles of IGFBPs are more complex than regulation of action of IGFs.

IGFBP-4 is N-glycosylated and is more resistant to proteolysis than the non-glycosylated form. Human IGFBP-4 has a molecular weight of 26 kDa and migrates as two isoforms on non-reducing SDS-PAGE at 28 kDa and 24 kDa. IGFBP-4 is present in human serum and has also been isolated from human bone cells, osteosarcoma cells, prostate cells, neuroblastoma cells and porcine ovarian follicular fluid (Ooi and Boisclair, 1999). IGFBP-4 is abundantly expressed in adult rat liver and to a much lesser extent in tissues such as the brain cortex,

hypothalamus, stomach, kidney, lung, heart, spleen, testis and adrenal gland. Embryonic expression of IGFBP-4 occurs in many tissues at mid and late gestation in rats. Pregnancy increases IGFBP-4 mRNA levels in liver at least 10 fold in rat. *In vitro* IGFBP-4 is synthesised by osteoblasts and acts to inhibit osteoblast proliferation. Parathyroid hormone treatment (3 hr) of osteoblast-like osteosarcoma cells (SaOS-2) increased the expression of IGFBP-4 mRNA and decreased IGFBP-4 protease activity (Kudo et al., 1997). Treatment of prepubertal pig sertoli cells with thyroid hormone (T_3) and retinoic acid increased their expression of IGFBP-4 mRNA in a dose dependent manner (Bardi et al., 1999). Proteolysis of IGFBP-4 is inhibited by binding of IGFs. IGF analogs that do not bind IGFBP-4 were found not to prevent proteolysis (Conover, 1999;Qin et al., 1999).

IGFBP-5 is a 252 amino acid protein with a molecular weight of 29 kDa. It is O-glycosylated and appears in nonreducing SDS-PAGE as three molecular forms 29, 32 and 43 kDa. The protein contains a string of Arg- Arg residues that allow binding to glycosaminoglycans and extra cellular matrix resulting in a 7 to12 fold decrease in binding affinity for IGF-I (Collett Solberg and Cohen, 1996). Phosphorylation of IGFBP-5 facilitates the connection to hydroxyapatite in bone matrix allowing preferentialy delivery of IGF-II for bone growth. IGFBP-5 is present in human serum. Recently IGFBP-5 has been demonstrated to bind ALS (Twigg et al., 2000) thus enabling formation of a ternary complex with IGF-I or II. This may be an alternative transport and storage system analogous to the 150 kDa complex containing IGFBP-3 or it may deliver IGFs to specific tissues. IGFBP-5 is expressed as early as day 10.5 of gestation in the rat embryo. In the adult rat IGFBP-5 mRNA is abundant in the kidney and white adipose tissue but is also present in lower amounts in skeletal muscle, brain, stomach, lung heart, adrenal, intestine, liver and spleen. Hypophysectomy reduces the abundance of IGFBP-5 mRNA in skeletal muscle and white adipose tissue which can be partially restored

with IGF-I and completely by treatment with GH (Ooi and Boisclair, 1999). Thus GH regulates IGFBP-5 similarly to IGFBP-3.

IGFBP-6 was first isolated from SV-40 transformed or nontransformed human lung fibroblasts and U-2 human osteosarcoma cells (Ooi and Boisclair, 1999). Human IGFBP-6 is O-glycosylated, has a molecular weight of 22.8 kDa and migrates on a nonreducing SDS-PAGE gel at 28 to 34 kDa. IGFBP-6 is found in human serum, CSF, and porcine follicular fluid. IGFBP-6 binds IGF-II with much greater affinity than IGF-I and the glycosylated form has a 5 fold higher affinity for IGF-I compared with the nonglycosylated form. Retinoic acid increased the expression of IGFBP-6 mRNA in cultured osteoblasts and the IGFBP-6 protein in the culture medium (Gabbitas et al., 1996). Abundance of IGFBP-6 mRNA in rat ovary is increased after hypophysectomy. Treatment with FSH decreased ovarian IGFBP-6 mRNA at least 2-fold (Rohan et al., 1993).

1.4.1.2 Proteases

IGFBP proteolytic activity was identified initially in serum of pregnant women (Gargosky et al., 1992;Hossenlopp et al., 1990) and in states of severe illness and other catabolic states. Proteases have been identified that modify other IGFBPs (Table 1.1). Human pregnancy serum proteases have the ability to degrade circulating IGFBPs. A serum protease of 70 to 90 kDa in size is present in pregnant and nonpregnant human serum and a larger 150 kDa protease was only present in pregnant serum (Bang and Fielder, 1997). Protease inhibitors aprotinin and α-2-antiplasmin inhibited proteolytic cleavage of radiolabelled IGFBP-3 when incubated with pregnancy serum suggesting that one of the proteases is plasmin. EDTA and zinc prevent the proteolytic degradation of radiolabelled IGFBP-3 indicating a cation dependent protease (Bang and Fielder, 1997). This protease was subsequently identified to be

a disintegrin metalloproteinase (Kubler et al., 1998). Human stomelysin-3 has been shown to degrade IGFBP-1 into two fragments 16 kDa and 9 kDa *in vitro* (Manes et al., 1997). All IGFBPs, except IGFBP-6, are proteolytically degraded by Cathepsin D (Claussen et al., 1997).

Human placental trophoblasts have been demonstrated to secrete an IGFBP-3 protease similar to that found in pregnant serum. Protease inhibitors EDTA, (4-(2-aminoethyl) benesulfonyl fluoride (AEBSF), aprotinin and o-phenanthroline prevented proteolysis of IGFBP-3 in pregnant serum and trophoblast conditioned medium. These inhibitors suggest the enzyme to be a disintegrin metalloproteinase (Irwin et al., 2000). IGFBP-5 proteolytic activity was identified in pregnant serum and amniotic fluid by the presence of fragments of 22 kDa and 15 kDa. EDTA, phenanthroline and PMSF inhibited this activity which is suggestive of a cation dependent serine protease, similar to proteases which degrade IGFBP-3 and IGFBP-2 (Claussen et al., 1994). Pregnancy-associated Plasma Protein-A2 has been identified as an IGFBP-5 protease (Overgaard et al., 2001). Proteolysis of the binding proteins may result in altered binding affinity for the IGFs, increased bioavailability of the IGFs and increased IGF-independent activity of IGFBPs.

Many proteases for which IGFBPs are substrates have been identified and these include plasmin, prostate-specific antigen, nerve growth factor, matrix metalloproteases, cathepsin D (Conover, 1999) with many yet to be defined. Regulation of IGFs and IGFBPs by proteases and the mechanisms involved remain to be elucidated, although specific tissue and cell proteases have the potential to convey precise and specific regulation of the IGF axis.

An acid protease in rat serum has been identified that cleaves IGF-I forming the des-(1-3) variant of IGF-I (Yamamoto and Murphy, 1994), which has very poor affinity for IGFBPs. The growth hormone-dependent serine protease inhibitor Spi 2.1 has been suggested as a

mechanism of regulating the acid protease responsible for generating des(1-3) IGF-I (Maake et al., 1997). In general proteases alter the bioavailablity of IGFs and may also generate variant forms with altered bioactivity.

Table 1.1 Insulin-like binding protein specific proteases

IGF-binding protein	Protease	IGFBP protease origin
IGFBP-1	Cathepsin D Stromolysis-3 (MMP-11) Plasmin	Decidua Amniotic fluid
IGFBP-2	Tissue plasminogen activator Cation-dependent serine protease	Newborn fasted pig Follicular fluid (dominant follicle) Human milk
IGFBP-3	Cation-dependent serine protease Plasmin disintegrin metalloproteinase	Pregnancy serum human Human placental trophoblast
IGFBP-4	pregnancy-associated plasma protein-A (PAPP-A)	Human pregnancy serum Ovarian follicular fluid Cultured adult human fibroblasts
IGFBP-5	Cation-dependent serine protease PAPP-A2	Human pregnancy serum Amiotic fluid

References:

(Bang and Fielder, 1997;Byun et al., 2001;Claussen et al., 1997;Claussen et al., 1994;Collett Solberg and Cohen, 1996;Conover et al., 1999;Irwin et al., 2000;Kubler et al., 1998;Lawrence et al., 1999;Manes et al., 1997;Overgaard et al., 2001;Rajah et al., 1995)

1.5 Regulation of insulin-like growth factor-I abundance

1.5.1 Growth hormone

Growth hormone promotes the expression of IGF-I in adult rat liver. Hypophysectomized rats have reduced abundance of hepatic IGF-I mRNA and reduced IGF-I in plasma. Growth

hormone treatment of hypophysectomized rats restores the levels of IGF-I mRNA to near normal (Roberts et al., 1986). Growth hormone also regulates the expression of IGF-I mRNA in pig adipocytes as well as rat heart and skeletal muscle (Coleman et al., 1994;Isgaard et al., 1989). In rats abundance of hepatic IGF-I mRNA correlates positively with circulating levels of IGF-I suggesting that the liver is the major source of endocrine IGF-I in this species. Rat liver has higher abundance of IGF-Ib mRNA than heart, lung, muscle, testes, stomach, kidney and brain. IGF-Ia mRNA abundance is similar in most tissues in adult rats. GH treatment increases both IGF-Ia mRNA and IGF-Ib mRNA simultaneously in many tissues. In liver, however, the increase in IGF-Ib mRNA in response to GH is much greater in rats (Lowe et al., 1988). The use of antibodies specific to the E peptide region EIa_{91-103} of the IGF-Ia prohormone revealed that human hepatoma cells secrete prohormone IGF-Ia in culture and human growth hormone increased the secretion in a dose dependent manner (Conover et al., 1993). Cell density has been reported to regulate the expression of IGF-I (Wang and Adamo, 2000). Adipose tissue has been suggested to be a major source of circulating IGF-I in both humans (Moller et al., 1991) and pigs (Coleman et al., 1994). Growth hormone regulation of IGF-I mRNA in hepatic and non-hepatic tissues suggests it controls endocrine, paracrine and autocrine actions of IGF-I.

The mechanism by which GH increases IGF-I expression has not been elucidated. However, potential GH responsive sites in the IGF-I gene promoter may be involved. GH binds to the GH receptor activating the expression of STAT-5, which is involved in insulin gene transcription. Growth hormone treatment of human hepatoma-derived Hep3B cells transfected with salmon IGF-I (sIGF-I) promoter-luciferase constructs simultaneously overexpressed both STAT5 and HNF-1α resulting in a 35 fold increase in sIGF-I promoter activity while STAT5 and HNF-1α alone showed no effect (Meton, 1999). This observation indicates that more than one transcription factor may be required to initiate IGF-I promoter

activity. Growth hormone treatment increased liver IGF-I mRNA expression in hypophysectomized wild type mice but this effect was not observed in STAT5b knockout mice (Davey et al., 2001). This indicates that STAT5b plays a significant role in GH induced IGF-I gene expression in the liver. A GH dependent DNAse I hypersensitive site, HS7, in the rat IGF-I gene has been identified. The DNAse sensitivity of the HS7 site disappeared after hypophysectomy of rats and was restored 30 mins after GH injections (Bichell et al., 1992;Rotwein et al., 1993). The mechanism by which GH alters chromatin structure in the IGF locus has yet to be defined.

1.5.2 Nutrition

Dietary energy restriction in adults and children reduces the concentration of IGF-I in blood while those of IGFBP-1 are increased in adults but not in children. Energy restriction did not alter IGFBP-2 in either group and blood levels of IGFBP-3 fell significantly in children but not in adults (Smith et al., 1995). Dietary protein restriction reduces circulating levels of IGF-I in both adults and children but increases those of IGFBP-2.

Fasting for 48 hr has been shown to reduce plasma IGF-I (Adamo et al., 1991a) and the expression of IGF-IEb, but not IGF-IEa transcripts, in rat liver (Zhang et al., 1997). Fasting resulted in the reduced abundance of 0.9 -1.2 kb and 1.5 -1.9 kb, but not the 7.5 kb, IGF-I mRNA species in rat liver. *In vitro* translation of liver mRNAs followed by immunoprecipitation with antibodies directed against pro-IGF-IEa, pro-IGF-IEb and the mature IGF-I peptide, indicated that fasting decreased both peptides and refeeding restored them to normal. The above results suggest fasting differentially regulates IGF-IEb and IGF-IEa mRNA from leader exon 1. A decline in translatable IGF-I mRNAs may account for the observed decrease in peptides, or alternatively efficiency of IGF-I mRNA translation was

lower. Reduced hepatic expression of IGF-I mRNA observed in fasted rats was not due to decreased IGF-I gene transcription, but rather a delay in pre-mRNA splicing, leading to an accumulation of IGF-I pre-mRNA in the cytoplasm (Zhang et al., 1998). The abundance of 0.9 - 1.2 kb, 1.5 -1.9 kb and 7.5 kb IGF-I mRNA species were all lower in rat hepatocytes cultured in amino acid deplete (5-fold) medium. The 7.5 kb IGF-I mRNA species degraded faster than the two smaller 0.9-1.2 kb and 1.5 -1.9 kb forms, suggesting protein restriction increases the rate of degradation of all the IGF-I mRNA species.

Nutrition and growth hormone have been shown to regulate the hepatic expression of leader exon class 1 and class 2 transcripts in sheep. Higher dietary protein and *ad libitum* feeding of lambs increased hepatic class 1 and class 2 transcripts although class 2 transcripts were 5-fold more abundant than class 1 in the liver. However growth hormone was shown to increase class 2 transcripts by more than 100% (Pell et al., 1993). Circulating concentrations of IGF-I were positively related to the ratio of class 2 to class 1 transcripts. Pell *et al* (1993) suggested that the ratio of the class 2 to class 1 transcripts somehow determines plasma IGF-I concentrations.

Growth hormone treatment of pigs increases hepatic abundance of leader exon class 1 transcripts 4-fold and class 2 transcripts 8-fold (Brameld et al., 1996;Dunaiski et al., 1999). Dietary protein composition had no effect on abundance of class 1 or class 2 transcripts in the porcine liver. However increased dietary protein levels or growth hormone treatment increased the expression of class 1 transcripts in semitendinous muscle, subcutaneous fat, perirenal fat and omental fat but not in longissimus dorsi muscle in this species (Brameld et al., 1996). In general in pigs regulation of liver IGF-I mRNA transcripts appears to be growth hormone dependent while nonhepatic IGF-I mRNA transcripts are regulated by nutrition and GH in a tissue specific manner.

Table 1.2 Growth hormone and nutritional responsiveness of the leader exons

Species	Leader Exon Class	Growth hormone	Increased nutrition dietary protein and *ad libitum* feeding
Rat	1	↑ 2-fold	
	2	↑ 7-fold	
Sheep	1	-	↑
	2	↑	↑
Pig	1	↑ 2-fold	↑
	2	↑ 8-fold	↑

References:

(Brameld et al., 1996;Dunshea et al., 1999;Ohlsen et al., 1993;Pell and Gilmour, 1993;Weller et al., 1993;Zhang et al., 1997)

1.5.3 Insulin

Young rats treated with streptozotocin to induce insulin deficiency grow poorly and have low levels of hepatic IGF-I mRNA and serum concentrations of IGF-I and GH (Fagin et al., 1989;Menon et al., 1994;Yang et al., 1990). Insulin deficiency in human subjects increases circulating levels of IGFBP-1, IGFBP-2 and increases proteolysis of IGFBP-3 (Bereket et al., 1999). Insulin administration to streptozotocin treated rats restores hepatic IGF-I mRNA and serum IGF-I concentrations to those of untreated normal rats (Yang et al., 1990;Zapf, 1998). Northern blot and ribonuclease protection assay showed that the expression of growth hormone receptor/growth hormone binding protein mRNA was reduced in liver and heart and increased in the kidney of insulin deficient rats. Insulin deficiency induces a GH resistance

state that is tissue specific (Menon et al., 1994). Growth hormone plasma levels are also elevated in insulin deficiency possibly due to a diminished negative feedback by IGF-I that normally inhibits GH expression (Bereket et al., 1999). Insulin treatment partially restored hepatic IGF-I mRNA and IGFBPs mRNA in food restricted hypoinsulinemic rats. However, insulin and re-feeding returned hepatic IGF-I mRNA and plasma IGFBPs concentrations back to that of control rats (Goya et al., 1999). The effect of insulin deficiency on IGF-I abundance may be altered by pregnancy. Insulin deficiency in pregnant pigs increased the abundance of IGF-I mRNA in fetal skeletal muscle, liver, heart, kidney and placenta and reduced it in fetal adipose and brain tissue (Ramsay et al., 1994).

1.5.4 Other Hormones

Estrogen and progesterone increase the abundance of IGF-I mRNA in female reproductive tissues. Gonadotrophins also increase expression of IGF-I mRNA in the ovary and testes. Thyroid hormone (T_3) in conjunction with GH stimulates the expression of IGF-I in cultured GH_3 rat pituitary cells and primary cultures of rat hepatocytes. Thyroidectomy of neonatal, weaning and adult rats decreases body weight. In the neonatal rat plasma insulin, plasma GH, pituitary GH, plasma IGF-I and hepatic IGF-I mRNA are increased by thyroidectomy. However, in weaning rats and adult rats removal of the thyroid decreased plasma GH, pituitary GH, plasma IGF-I and hepatic IGF-I mRNA (Ramos et al., 1998). This indicates developmental regulation of IGF-I response to thyroid hormones in the rat.

Parathyroid hormone (PTH) is a trophic factor for bone and has been shown to increase the abundance of IGF-I mRNA in cultured preosteoblast cells. PTH also decreased the abundance of IGFBP-3 mRNA, with no significant effect on IGF-I mRNA.

Infusion of cortisol into adrenalectomized fetal sheep late in gestation increased hepatic abundance of IGF-I mRNA and GH receptor mRNA. Positive relationships were found between plasma concentrations of cortisol, total hepatic IGF-I mRNA and the abundance of leader exon class 1 and class 2 IGF-I transcripts (Li et al., 1996). Dexamethasone, a synthetic glucocorticoid, decreases GH receptor mRNA in cultured rat chondrocytes thereby inhibiting GH stimulation of IGF-I in a dose and time dependent manner (Jux et al., 1998). Preincubation of osteoblasts with dexamethasone increased the ability of low concentrations of prostaglandins (PGE_2) to actively stimulate the IGF-I promoter, however high or sustained levels of PGE_2 inhibit IGF-I expression (McCarthy et al., 2000).

1.5.5 Developmental Regulation

Synthesis of IGF-I and IGF-II are developmentally regulated. IGF-I mRNA transcripts have been identified in the preimplantation mouse embryo and uterus (Doherty et al., 1994;Henemyre and Markoff, 1998). In the rat embryo expression of IGF-I mRNA increases 9-fold between days 11 to 13 of gestation (Adamo et al., 1989;Bondy et al., 1990). In humans circulating IGF-I levels are low in fetal life, increase progressively with age from birth, peak during puberty and slowly decline with increasing age. In rats liver IGF-I mRNA abundance increases 10 to 100-fold from birth to adulthood and correlates with plasma IGF-I suggesting that the liver is the major endocrine source of IGF-I peptide (Hoyt et al., 1988).
The Cre/loxP system has been used to abolish IGF-I gene expression in the murine liver. This resulted in a 75% reduction in plasma IGF-I and IGFBP-3 confirming the liver as a major source of endocrine IGF-I in the nonpregnant mice (Le Roith et al., 2001;Yakar et al., 1999). However these liver-specific IGF-I knockout mice grow normally suggesting liver derived IGF-I is not essential for postnatal growth in mice.

Developmentally the leader exon class 1 transcripts increase from birth in the rat, whereas class 2 transcripts do not appear until 20-30 days postnatally, consistent with the appearance of GH receptors in liver of this species (Adamo et al., 1989;Hoyt et al., 1988). However in the sheep expression of hepatic class 1 and class 2 IGF-I mRNA transcripts increased from 100 to 145 days gestation in the fetus (Li et al., 1996). Leader exon class 1and class 2 transcripts are also present in ovine fetal and adult brain, heart, kidney, liver, lung, skeletal muscle and spleen (Ohlsen et al., 1993). Adrenalectomy of fetal sheep at ~ day 117 (term is ~ 145 d) decreased hepatic expression of IGF-I class 1 and class 2 transcripts (Li et al., 1996).

Leader exon class 1 IGF-IEa transcripts increase in abundance in rat liver from birth to 30 days of age. In rats the stomach, brain, lung, kidney, ovary and testes of fetal and adult origin (Hoyt et al., 1988;Murphy et al., 1987) also express IGF-IEa class 1 transcripts. However adult rat liver expresses class 1and class 2 transcripts encoding the IGF-IEa E domain and class 1 and class 2 transcripts encoding the IGF-IEb E domain. Non hepatic synthesis of IGF-I mRNA would suggest autocrine and paracrine action pre and postnatally.

In human fetal tissues of 10 to 16 weeks gestation IGFBP-1 mRNA was found to be expressed in the liver, IGFBP-2 mRNA was moderately expressed in every tissue examined with the highest levels in the liver, IGFBP-3 was abundantly expressed in the skin, muscle and heart while IGFBP-4 was equally expressed in all tissues. In fetal pigs the abundance of IGFBP-2 mRNA in the liver and concentrations of plasma IGFBP-2 increase with gestation but decline postnatally. Plasma levels of IGF-I and IGFBP-3 are low in the fetal pig but increase postnatally (Lee et al., 1991;Lee et al., 1993). The abundance of IGF-I mRNA and IGFBP-3 mRNA in skeletal muscle, lung and kidney was similar to that in liver of fetal pigs. Their expression postnatally did not increase in contrast to that seen in rats. Differences in the type

and abundance of IGF-I transcripts in both fetal and adult tissues indicate complex regulation of IGF-I synthesis.

Plasma levels of IGF-II in rats are 20-100 fold lower at day 21 of age than those at birth (Brown et al., 1986) and IGF-II mRNA is much more abundant in fetal rat tissue compared to adult tissue. Many fetal tissues including intestine, muscle, skin and heart express IGF-II up to 11 days after birth whereas liver IGF-II mRNA persists to day 22 in rats. Expression of IGF-II completely disappears from the lungs, thymus and kidney 2 days after birth (Brown et al., 1986;Lund et al., 1986). The cerebral cortex and hypothalamus (Hynes et al., 1988) are the only adult tissues to consistently express IGF-II mRNA in adult rats suggesting a role for IGF-II in the central nervous system. Concentrations of IGF-II in serum and plasma of adult rats are negligible. In guinea pigs serum IGF-II concentrations in adults are higher than those of IGF-I as is the case in human, pig, sheep and cow. Abundance of IGF-II mRNA is 30 times greater in fetal guinea pig liver, 18 times greater in skeletal muscle and 5 times greater in the kidney compared with adult guinea pig liver. Adult guinea pig tissues persistently express IGF-II mRNA, although it is 5 times more abundant in skeletal muscle than liver (Levinovitz et al., 1992).

1.6 Biological activity of Insulin-like growth factors

1.6.1 *In-vitro*

Cell proliferation

IGFs are mitogenic for a wide number of cell types in culture including fibroblasts, chondrocytes, osteoblasts, keratinocytes, thyroid follicular cells, mammary epithelial cells, erythroid progenitor cells, thymic epithelium, oocytes, granulosa cells, spermatogonia, sertoli cells and a number of cancer cell lines. These actions are generally mediated by type I IGF receptors (Jones and Clemmons, 1995).

Cell differentiation

IGFs also promote differentiation of many cell types. Treatment of cultured myoblasts with IGF-I increases myogenin mRNA abundance and has been associated with differentiation to myotubes (Florini et al., 1996). Myoblast differentiation was inhibited by a phosphatidylinositol (PI) 3-kinase inhibitor LY294002 (Tureckova et al., 2001). IGF-I activation of the type I IGF receptor can stimulate proliferation and differentiation by activating distinct signalling pathways in myoblasts. LY294002 inhibited PI_3-kinase/$p70^{S6k}$ abolishing myotube formation. Inhibition of MEK in the MAP-kinase pathway by PD098059 inhibitor stimulated differentiation of cultured L6A1 myoblasts (Coolican et al., 1997). IGF stimulation of myogenic differentiation requires the activation of the phosphatidylinositol 3-kinase/$p70^{S6k}$ pathway (Coolican et al., 1997). IGF-I promotes differentiation of secondary spermatogonia into primary spermatocytes (Yamamoto et al., 2001). Primary cultures of preadipocytes and cultured 3T3-L1 preadipocytes when treated with IGF-I undergo differentiation when cells reach confluency (Boney et al., 1998).

Apoptosis

IGFs stimulate both proliferation and differentiation and also protect cells from apoptosis (Butt et al., 1999). Overexpression of IGF-I in the cental nervous system of transgenic mice reduces the number of apoptotic cells in 7, 14 and 21 day old mice. This was associated with an increase in anti-apoptotic Bcl genes, Bcl-x(L) and Bcl-2 and a concomitant decrease in pro-apoptotic Bcl genes, Bax and Bad by 21 days postnatally (Chrysis et al., 2001). IGF-I increases the number of human embryos forming blastocysts and decreases the number of apoptotic nuclei measured by DNA fragmentation (TUNEL) and nuclear fragmentation (Spanos et al., 2000). However high levels of IGF-I and insulin stimulate apoptosis in the mouse blastocyst inner cell mass. It has been suggested that high levels of IGF-I decrease the abundance of the type I IGF receptor preventing activation of the anti-apoptotic signalling cascade.

IGFBP-3 also plays a role in regulating apoptosis by sequestering IGFs away from the type 1 receptor and /or by independent action with cell surface receptors for IGFBP-3. Cultured Hs578T breast carcinoma cells proteolytically fragment IGFBP-3 and increase ceramide induced apoptosis. However this effect was inhibited by IGF-I which prevented proteolytic degradation and cell surface association. Hs578T breast carcinoma cells are unusual in that they lack the type I IGF receptor and do not secrete detectable amounts of IGF-I or IGF-II. IGFBP-3 appears to be proteolysed on the surface of these cells and this mechanism then predisposes the Hs578T cells to enhanced ceramide apoptosis (Maile et al., 1999). A critical balance between interaction of IGF-I and IGFBP-3 and subsequent activation of anti-apoptotic and pro-apoptotic transduction pathway genes appears to be the mechanism controlling apoptosis. Actions of IGF-I promoting cellular proliferation and IGFBP-3's ability to inhibit

or sequester IGF-I can contribute to the survival of mutated cells and progression to tumours (Holly et al., 1999).

Cell metabolism

Some of the biological actions of IGF-I and IGF-II are similar to those of insulin. In rat adipocytes the IGFs and insulin increase glucose uptake, glucose oxidation and lipid synthesis and inhibit lypolysis and glycogen synthesis. Neutralization of the insulin receptor by anti-insulin receptor antibodies demonstrated that the IGFs actions were mediated by the insulin receptor in the rat adipocyte (Zapf, 1999). IGF-I and IGF-II are 50 to 100 times less potent than insulin in this action. However, in isolated soleus muscle cells competitive binding studies showed that IGF actions are mediated via the IGF-I receptor. These actions include stimulation of glucose uptake, glycogen synthesis as well as increased amino acid uptake and protein synthesis. (Zapf, 1999).

1.6.2 *In-vivo*

Administered IGF-I

In normal human subjects infusion of IGF-I has been shown to increase glucose uptake producing hypoglycemia. Plasma insulin levels are also reduced. IGF-I infusion at 0.4 µg /kg per min over 3 hours with a constant infusion of glucose increased [^{3}H] glucose uptake, and reduced plasma concentrations of amino acids and free fatty acids (Boulware et al., 1992;Sherwin et al., 1994). Rats receiving a 4 hour infusion of IGF-I (1 µg/kg per min) supplemented with amino acids had higher rates of protein synthesis in the gastrocnemius, oblique and soleus muscle (Jacob et al., 1996). IGF-I acutely inhibits muscle protein degradation (Oddy and Owens, 1996). Infusion of IGF-I into normal rats has been shown to increase growth through an increase in nitrogen retention. IGF-I at 1.2 or 2.9 mg/kg per day for seven days was able to improve nitrogen retention in protein restricted rats (Tomas et al.,

1991). Organ weights in these animals were not altered except for the kidneys, which increased at the higher dose of IGF-I. Hypophysectomized female rats have been infused with IGF-I for 6 days (1.25 mg/kg per day). After 6 days body weight, spleen weight and plasma IGF-I increased. IGF-I treatment decreased insulin stimulated ^{14}C-glucose incorporation into isolated rat adipocytes which was probably due to the reduced abundance of insulin receptors secondary to decreased plasma insulin levels caused by IGF-I treatment (Frick et al., 2000). IGF-I increased glucose incorporation into glycogen in soleus muscle of hypophysectomized female rats. Exercise induced protein synthesis was reduced by a neutralizing anti-IGF-I antibody in diabetic rats (Fedele et al., 2001). IGF-I treatment of insulin deficient rats restored glucose transporters Glut 2 and Glut 4 back to that of the normal rat (Asada et al., 1998).

Fetal sheep treated with IGF-I (26 μg/h.kg) between day 120 to 130 of gestation had higher weights of several organs including liver, lung, heart, kidneys spleen adrenals and pituitary (Lok et al., 1996). IGF-I treatment did not significantly affect total placental weight and average placentomes weight but increased placentome in the weight range of 12 to 13.9 g. Infused IGF-I increased the cross sectional area of long bones while length was unaffected. IGF-I therefore has specific effects on fetal organ development, endocrine glands and skeletal growth in fetal sheep. Hepatic abundance of IGF-I mRNA was decreased in fetal liver, but not skeletal muscle, suggesting administered IGF-I negatively regulates hepatic expression of IGF-I. Conversely fetal plasma levels of IGFBP-1 and IGFBP-3 were increased by IGF-I treatment (Kind et al., 1996) suggesting IGF-I may play a role in regulating hepatic synthesis of IGFBP-1 and -3. IGF-I at 9.9, 20.1, 40.2 nmol/h infused from 122 to 127 days in ovine fetuses increased glucose uptake, while plasma insulin was decreased at the two higher doses. IGF-I treatment of the ovine fetus increased amino acid metabolism, as indicated by the increased transfer of phenylalanine from the mother to the fetus and decrease in amino acid

loss from the fetus to the placenta (Liechty et al., 1999). IGF-I acutely increases glucose uptake by muscle and reduces degradation of muscle protein regardless of nutrition (Oddy and Owens, 1996).

Gene knockout

Null mutations of the genes for IGF-I, IGF-II, type 1 IGF receptor, type 2 IGF receptor and multiple gene deletions have further elucidated their roles in developmental biology of the mouse (Table 1.3). Expression of IGF-I and the type I IGF receptor are both required for normal fetal growth (Powell-Braxton et al., 1993) as IGF-I gene deletion results in birth weight that is ~60% that of normal mice. Placental growth was unaffected but perinatal survival and postnatal growth is very poor in IGF-I gene knockout mice. Gene deletion of the type I IGF receptor affects fetal growth as early as ~10.5 days gestation, reducing birth weight to ~45% of normal wild type mice. Mice lacking the type I IGF receptor do not survive. IGF-II gene deletion affects intrauterine growth and reduces birth weight by ~40%. Placental growth is decreased in IGF-II knock out mice similar to that seen in type II IGF receptor knockout mice (Baker et al., 1993), suggesting placental development is regulated by IGF-II and the type II IGF receptor. A type II IGF receptor deletion results in an increase in birth weight of ~35 to 40% and reduced placental development from day 13 of gestation. IGF-II levels are increased in this experimental animal promoting overgrowth of the fetus, possibly acting through the type I IGF receptor (Morison et al., 1996). Insulin receptor gene deletion reduced fetal growth from ~18.5 days of gestation resulting in birth weight ~90% that of wild type mice. Placental development was unaffected, however, none survived possibly due to the effects of diabetes.

Mutation	Fetal Growth		Placental Growth		Birth Weight (~% normal)	Survival Rate	Postnatal Growth
	nature	onset	nature	onset			
IGF-I	↓	~13	none		~60	5-70%	very slow
IGF-II	↓	~10.5	↓	13	~60	normal	normal rate
type I IGF receptor	↓	~10.5	none		~45	none	-
type II IGF receptor	↑	~13	↓	13	135 - 40	poor	
Insulin receptor	↓	~18.5	none		~90	none	-
type I IGF, type II IGF receptors			↑	13	~100	normal	slower than normal
IGF-II, type I IGF, type II IGF receptors					~35	none	none
IGF-II,type II IGF receptors					~100	normal	slightly below normal
Insulin receptor, type I IGF	↓			none	~30	none	none

Table 1.3 Growth, survival and birth weight characteristics of knockout mice with single or multiple gene deletions of the IGF system

In multiple gene knockout mice, where both type I IGF receptor and type II IGF receptor are deleted, birth weight, fetal growth was near normal and placental growth was increased. This suggests that fetal growth and placental growth can be mediated by another receptor that is normally redundant. This is probably the insulin receptor. In mice lacking either type I IGF receptor and/or type II IGF receptor, plasma IGF-I and IGF-II are increased. This may be necessary before signalling occurs through an alternate receptor, possibly the insulin receptor. In double knockout mice, lacking both the insulin receptor and type I IGF I receptor, fetal growth is more severely reduced than the single gene deletion of type I IGF receptor. Placental growth was unaffected in the insulin receptor and type I IGF receptor double knockout mice. This indicates that IGF-II acts through the type II IGF receptor to promote placental growth. Gene deletion of both IGF-II and type II IGF receptor reduced birth weight by ~75% but postnatal growth rate (fractional) and survival was almost normal. Triple gene ablation of IGF-II, type I IGF receptor and type II IGF receptor caused a 65 % reduction in birth weight with none surviving.

Null mutation of IGFBPs did not result in major phenotypic alterations and this is probably due to other IGFBPs compensating. IGFBP-2 gene deletion decreased spleen weight in adult males (Wood et al., 1993). IGFBP-4 null mutation resulted in slightly smaller mice.

Overexpression of IGFs and IGFBPs in transgenic mice

Several transgenic mice lines over expressing IGF-I driven by different promoters have been developed. The tissue that expresses the IGF-I transgene determines the effects of IGF-I trangenesis. IGF-I transgenic mice driven by the mouse metallothionein promoter overexpress IGF-I in many tissues, except the liver. Weights of brain, pancreas, kidney, spleen and carcass are increased, however, bone growth was unaffected in these IGF-I mice. IGF-I transgenic

mice crossed with GH deficient mice provided further evidence for a GH mediated IGF-I action on bone growth and somatic growth (Behringer et al., 1990). This transgenic model highlights IGF-I independent actions as brain size was increased and liver weight decreased. Skeletal muscle overexpression of IGF-I results in myofibril hypertrophy. In transgenic mice expressing IGF-II under the control of the rat PEPCK promoter IGF-II was expressed in liver, kidney and several parts of the gastrointestinal tract, increasing plasma IGF-II concentrations by 2 to 3 fold in 4 and 12 week old mice. There were no noticeable changes in body weight in 4 week old transgenic animals, but kidney and testis weights were increased at 4 weeks of age while adrenal weight was increased at 12 weeks of age (Wolf et al., 1994).

Many transgenic mice expressing IGFBP transgenes have been developed (Schneider et al., 2000). Two IGFBP-1 transgenic mice lines have been developed, one controlled by metallothionein promoter MT-hIGFBP-1 and another by the phosphoglycerate kinase promoter PGK-rIGFBP-1. Transgenic mice overexpressing IGFBP-1 were characterised by reduced somatic growth, reduced birth weight (83 to 92 % of the wild type), altered glucose homeostasis and altered pancreatic structure (Rajkumar et al., 1999). In IGFBP-2 transgenic mice controlled by the cytomegalovirus promoter, the transgene was expressed in pancreas, stomach, skeletal muscle, heart, colon, spleen, adipose tissue, brain and kidney. Overexpression of IGFBP-2 resulted in a 3-fold increase in plasma IGFBP-2, decreased carcass weight, body weight and spleen weight (Hoeflich et al., 1999). IGFBP-3 transgenic mice driven by the mouse MT promoter express the IGFBP-3 transgene in the kidney, small intestine, and colon (Murphy et al., 1995). The spleen, liver and heart were enlarged in IGFBP-3 transgenic mice.

1.7 Insulin-like growth factors in pregnancy

1.7.1 Effect of pregnancy on the endocrine IGF system

In rats plasma IGF-I is increased from early to mid pregnancy then progressively declines from day 14 to term at day 21 (Davenport et al., 1990;Gargosky et al., 1990b;Travers et al., 1993). IGF-I protein concentrations in liver are lower on day 21 of pregnancy compared with day 4 postpartum and non-pregnant rats. A decline in IGF-I synthesis by liver therefore appears responsible for decreased serum concentrations (Davenport et al., 1990). IGF-II is found circulating in adults of most mammalian species, except for rats and mice where IGF-II is not present in adult plasma (Daughaday and Rotwein, 1989). Pregnancy increases plasma levels of IGF-II in humans, rabbits and baboons (Gargosky et al., 1995;Giudice et al., 1990;Nason et al., 1996).

Early studies show that pregnancy does not affect plasma IGF-I and IGF-II in guinea pigs (Daughaday et al., 1986). However, after removal of IGF-binding proteins from guinea pig plasma before assay it was found that IGF-I levels in blood were elevated by pregnancy in this species (Sohlstrom et al., 1998). Pregnancy did not alter circulating IGF-II in guinea pigs (Sohlstrom et al., 1998). Pregnancy increased plasma IGFBP-4, but not IGFBP-3, in guinea pigs.

Plasma levels of IGFBP-3 are increased 10 fold by pregnancy in baboons (Giudice et al., 1993). IGFBP-3 normally circulates as two molecular forms in nonpregnant baboon plasma, one of 40 to 45 kDa and another of 26 to 28 kDa. In pregnancy a third molecular form of 18 kDa appears. On further investigation it was found that pregnant baboon decidua produced

identical molecular forms of IGFBP-3 to that found in pregnant serum. In many species proteolysis of plasma IGFBP-3 has not been demonstrated. Tissue specific proteases may be effective in posttranslational modification of IGFBPs as seen in human decidua (section 1.4.2).

Plasma levels of IGFBP-3, as detected by western ligand blotting, were found to be very low between days 12 to 21 of pregnancy in rats (Davenport et al., 1990;Gargosky et al., 1990b). Incubation of pregnant rat serum with 25mM antipain, which is known to inhibit serine and cysteine proteases, inhibited proteolytic fragmentation of IGFBP-3 by serum from pregnant rats. These pregnancy-induced proteases are thought to alter the bioavailability and bioactivity of endocrine IGFs. Protease activity decreases IGFBP-3 affinity for IGFs thereby increasing the clearance of IGFs from the circulation of pregnant rats (Bastian et al., 1993;Davenport et al., 1990). Radiolabelled I^{125}IGF-I was cleared 3 to 5 times faster in pregnant rats than nonpregnant rats. Increased clearance was accompanied by an increased uptake of I^{125}IGF-I by tissues (Bastian et al., 1993). Radiolabelled I^{125}IGF-I was detected in the placenta, fetus and fetal plasma (Bastian et al., 1993). It is also been reported that IGFBP-3 fragments bind to IGFBP-3 receptors on the cell surface (Baxter, 2000). This may alter IGF-I presentation to cell receptors increasing its delivery to certain cells. Proteolysed IGFBP-3 fragments retain the ability to bind IGFs and the ALS, but with reduced affinity (Suikkari and Baxter, 1992).

IGFBP-3 at concentrations of 100 to 300 pmol did not inhibit binding of insulin to a crude preparation of human placental microsomal membranes, however, IGFBP-7/mac25 inhibited 60 to 90% of the binding at the respective concentrations. The ability of IGFBPs to bind insulin and to prevent insulin binding to insulin receptors suggests a potential role in

maintaining insulin resistance states as seen in pregnancy and diabetes (Yamanaka et al., 1997).

Pregnancy also increases serum IGF-I in humans. Plasma IGF-I reaches maximum concentrations between 33 and 38 weeks (Gargosky et al., 1990a). Pregnancy decreases IGFBP-3, IGFBP-2, and IGFBP-4 and increases IGFBP-1 in the human as seen by western ligand blotting. The 45 kDa form of IGFBP-3 in human serum seen in western ligand blots disappeared during pregnancy similar to that observed in the rat. However, immunoreactive IGFBP-3 was unaltered by pregnancy in humans. Proteolysis changes the affinity of IGFBP-3 for radiolabelled IGF-I, but not for native IGF-I (Gargosky et al., 1992;Suikkari and Baxter, 1992).

IGFBP-1 is increased in mid and late pregnancy in the human (Giudice et al., 1990), baboon (Gargosky et al., 1995), rhesus monkey (Tarantal and Gargosky, 1995), rat (Donovan et al., 1991) and guinea pig (Sohlstrom et al., 1998). The ratio of highly phosphorylated isoforms to low phosphorylated isoforms of IGFBP-1 was negatively related to birth weight in normal and type I diabetic patients (Gibson et al., 1999). Low levels of phosphorylated isoforms were positively related to maternal weight through gestation and initial body mass index in diabetic subjects (Gibson et al., 1999). Maternal IGFBP-1 levels are higher in mothers carrying a fetus that is small for gestational age and lower for a large fetus (Hills et al., 1996). The degree of phosphorylation of IGFBP-1 carried in pregnancy may be potential regulators of the actions of IGF-I and –II on maternal metabolism. IGFBP-3 and IGFBP-1 both regulate the hypoglycemic activity of the IGFs and this characteristic may be important in protecting pregnant women from the adverse effect of increased IGF-I on glucose metabolism (Baxter, 1995). Glucose sparing in the mother occurs to ensure a supply of substrate for fetal growth.

The IGFs and IGFBPs can be thought of as a fine-tuning mechanism regulating circulating glucose levels (Bang et al., 1994).

Table 1.4 Effect of pregnancy on maternal endocrine IGF system

Species	Plasma IGF-I	Plasma IGF-II	Plasma IGFBP-3
Rat/ Mouse	↑12-13d ↓14-21d	undetectable	↓ by WLB + proteolysis
Guinea pig	↑-69d	no change	no change by WLB - proteolysis
Rabbit	↑-22d ↓22-30d	↑12-22d	↑12-28 - proteolysis
Sheep	no change		
Pig	24% ↓ from 60 -75 d 56% ↓ 112 d	27% ↓ from 45 d to term	↓ from 75 d to term on WLB
Cow	↓ from 148 to 253 d ↑ to 274 d	↑ from 85 to 148 ↓ up to 190 ↑ up to 21 and ↓ to 253 ↑ to 274 d	
Monkey	no change	no change	↑
Rhesus monkey	no change	↑	- proteolysis
Baboon	no change	↑ 2-fold	↑ 10-fold - proteolysis
Human	↑peak at 33-38 wk	↑ peaks 27-30 wk	↓by WLB + proteolysis

↑ increase, ↓decrease, WLB western ligand blotting, - no proteases activity in blood,

References: (Daughaday et al., 1986;Gargosky et al., 1995;Gargosky et al., 1990a;Gargosky et al., 1990b;Giudice et al., 1993;Hossner et al., 1997;Nason et al., 1996;Sohlstrom et al., 1998;Tarantal and Gargosky, 1995;Travers et al., 1990;Van Vliet et al., 1983).

1.7.2 Effect of pregnancy on expression of IGF-I, IGF-II and IGFBPs in liver

The abundance of all size classes of IGF-I mRNA transcripts (0.9 -1.2 kb, 1.5-1.9 kb, 4.7 kb, 7.5 kb) are reported to be lower in livers from 21 d pregnant rats than those from normal rats (Davenport et al., 1990) although in another study the 0.9 -1.2 kb transcript was unaffected (Travers et al., 1993). When IGF-I mRNA data was corrected for liver weight and total RNA content, total body content of hepatic IGF-I mRNA content did not decrease in pregnancy. The abundance of liver IGF-I mRNA was also unaffected by a 40% reduction in food intake in pregnant rats (Monaco and Donovan, 1996).

Pregnancy did not affect the hepatic abundance of IGFBP-3 mRNA in rats (Donovan et al., 1991). Food restriction increased expression of hepatic IGFBP-1 mRNA in pregnant and nonpregnant rats (Monaco and Donovan, 1996). There was no effect of pregnancy or feed restriction on hepatic abundance of IGFBP-4 in rats.

The major source of elevated endocrine IGF-I in pregnancy has yet to be determined. Proteolytic modification of IGFBP-3 appears to account for the decline in plasma IGF-I late in rat pregnancy. However in humans and guinea pigs plasma concentrations of IGF-I remain elevated throughout pregnancy despite the presence of proteases for IGFBP-3 in human. Factors responsible for the increase of endocrine IGF-I in pregnancy have not been determined.

1.7.3 Effect of nutrition during pregnancy on endocrine IGFs

The timing, duration and degree of food restriction during pregnancy determine the response of serum IGF-I concentrations and liver IGF-I mRNA abundance in the mother and on the growth of the fetus. Fasting in mid pregnancy reduces circulating IGF-I, but not IGF-II, in guinea pigs (Jones et al., 1990). Pregnant rats fed 30% less than *ad libitum* for the first 10 days of pregnancy, followed by 40% less thereafter, had 77% lower plasma IGF-I from day 5 to day 20 of pregnancy (Monaco and Donovan, 1996). This food restriction significantly decreased fetal and placental weight, lowered body weight gain of mothers, but did not alter litter size at term. Hepatic IGF-I mRNA was unaffected by food restriction in pregnant rats (Monaco and Donovan, 1996). Thus the reduced circulating IGF-I levels may be due to increased clearance from blood because of proteolytic modifications of binding proteins, especially IGFBP-3, in rats.

Restriction of dietary protein intake either throughout or during the last week of pregnancy in rats decreases maternal plasma IGF-I and birth weight (Muaku et al., 1995a;Muaku et al., 1995b). However, dietary protein restriction through the first two weeks of pregnancy in rats had no effect on birth weight. In the mother weight gain, liver weight, plasma IGF-I, total hepatic IGF-I mRNA and all classes of IGF-I mRNA transcripts were reduced with the 7.5 kb transcript being most affected (Muaku et al., 1995b). Protein deprivation early in pregnancy in rats restricts maternal anabolism, therefore reducing maternal protein stores and fetal growth. Continuation of protein restriction late in pregnancy in rats results in severe retardation of fetal growth. Restoration of a normal protein diet in the last two weeks of pregnancy was sufficient to prevent fetal growth restriction in rats. Pregnant rats fed a low protein diet with the same calorie intake as those on a normal protein diet had lower plasma

IGF-I concentrations and hepatic IGF-I mRNA. Pups of mothers fed a low protein diet with the same caloric intake as control animals had lower birth weight and smaller livers (Muaku et al., 1995a). Protein content of the mothers diet therefore plays an important role in fetal growth in rats by regulating abundance of IGF-I mRNA in maternal liver and blood levels of IGF-I in the mother.

In guinea pigs, plasma IGF-I is increased by pregnancy in both feed restricted and *ad libitum* fed guinea pigs (Dwyer and Stickland, 1992;Sohlstrom et al., 1998). The duration of reduced food intake is important in regulating IGF-II during pregnancy in guinea pigs. Chronic feed restriction (~30% for ~12 weeks) reduces plasma IGF-II concentrations but pregnancy has no effect in guinea pigs (Sohlstrom et al., 1998).

1.7.4 Treatment with growth hormone during pregnancy

Infusion of growth hormone 2 μg/g body weight/day for 10 days during the last half of pregnancy into *ad libitum* fed rats increased maternal plasma IGF-I, maternal weight gain, spleen and carcass fractional weight (carcass weight as a % of body weight) (Woodall et al., 1999). Fetal and placental weights were unaffected. However growth hormone treatment (100 μg/rat per day for three days) late in pregnancy did not affect maternal plasma concentrations of IGF-I in *ad libitum* fed animals (Nakago et al., 1999). Maternal nitrogen retention is increased early in pregnancy in rats which was positively related to maternal plasma IGF-I, suggesting IGF-I has anabolic effects on mothers in early pregnancy. Gilts treated with growth hormone (5 mg/day) in the second quarter of pregnancy had higher plasma IGF-I, higher hepatic IGF-I mRNA and greater fetal and placental weights (Sterle et al., 1998;Sterle et al., 1995). Growth hormone treatment of pregnant pigs fed 30 % less than *ad libitum* have reduced back fat and increased maternal plasma IGF-I, fetal body weight,

liver weight and skull width. Pregnant ewes treated with 0.1 mg/kg of bovine GH twice a day for 10 days from 125 days (term is ~145d) had twice the normal level of plasma IGF-I. GH increased placental uptake of glucose 3-O-[methyl-^{3}H] and [^{14}C] urea but did not change fetal and placental weight (Harding et al., 1997). These studies suggest that growth hormone administered in pregnancy affects maternal metabolism either directly or indirectly through increases in maternal IGF-I.

1.7.5 Treatment with IGF-I during pregnancy

Rats infused during the 2nd half of pregnancy with IGF-I at 1.4 mg /kg body weight per day over 10 days had higher maternal weight gain and plasma IGF-I concentrations were almost twice those of vehicle treated rats. Fetal and placental weights were unaffected (Gargosky et al., 1991). However administration of IGF-I at 2 mg/kg body weight per day to feed restricted pregnant rats between days 10 to 20 failed to increase maternal plasma IGF-I, maternal body weight gain nor weights of maternal liver, heart, spleen, kidney and carcass nor fetal weight and placental weight (Woodall et al., 1999). Since IGF-I infused in the 2nd half of pregnancy in rats had anabolic effects on the mother. This may suggest the infused IGF-I was preferentially delivered to maternal tissues and not the fetus or placenta.

Ewes at 129 to 133 days of pregnancy infused with IGF-I (30 μg/kg.h) over 4 hrs had higher plasma IGF-I and higher maternal and fetal blood glucose concentrations. IGF-I also acutely elevates blood glucose in nonpregnant sheep by reducing insulin levels and hence increasing hepatic glucose production (Oddy and Owens, 1996). Placental lactate production and fetal lactate uptake were increased by IGF-I while amino nitrogen levels fell slightly in both mother and fetus (Liu et al., 1994). This study suggested that the ratio of maternal IGF-I to fetal IGF-I late in pregnancy might regulate substrate partitioning of glucose, lactate and amino acids

between mother and conceptus. Intrauterine growth retarded fetuses have lower plasma IGF-I levels than normal fetuses. Increased glucose delivery to the fetus could increase fetal production of IGF-I.

IGF-I infusion at 50 μg/h per kg into the ovine fetus decreased umbilical blood levels of serine and glycine while fetal uptake of serine and glycine was unaltered. However fetal hindlimb uptake of amino acids was decreased (Jensen et al., 2000). IGF-I appears to regulate amino acid distribution to selected tissues in the fetus. Infusion of IGF-I at 26 μg/h per kg into fetal sheep between 120 days to 130 days of gestation increased plasma IGF-I concentrations and weights of selective organs, including liver, lungs, heart, kidneys, spleen, pituitary and adrenal glands (Lok et al., 1996). IGF-I treatment of fetal sheep was shown to increase the cross sectional area of epiphysial areas in fetal bones. Administration of IGF-I (40 nmol/h) for 8 hours into a 130 day old ovine fetus increased circulating levels of IGF-I, IGFBP-1, IGFBP-3 and hepatic abundance of IGFBP-1 mRNA (Shen et al., 2001). Co-infusion of IGF-I and insulin reduced the effect on IGFBP-1 and IGBP-3 seen by IGF-I alone.

Increases in IGFBP-1 and –3 in fetal plasma following infusion of IGF-I suggests that binding proteins are either preventing IGF-I induced hypoglycaemia by inhibiting IGF-I actions or that IGFBP-1 and –3 bind IGF-I and deliver it to selective tissues. Alternatively the pool of free IGF-I acts to promote anabolic growth of selective organs and skeletal maturation (Lok et al., 1996).

1.8 Aims of the study

In general the aim of this study was to determine if the liver was the major source of endocrine IGF-I in pregnancy and the effect of pregnancy on associations between hepatic abundance of IGF-I mRNA, plasma IGFs and pregnancy phenotype.

Experiments were designed to test the following hypotheses

1. Pregnancy increases the abundance of IGF-I mRNA in the liver and the concentration of IGF-I protein in blood in guinea pigs.

2. Guinea pig liver is the major source of endocrine IGF-I in pregnancy.

3. The circulating levels of IGF-I and hepatic abundance of IGF-I mRNA in the mother are related to the weights of the conceptus in late pregnancy

4. Insulin-like binding proteins in maternal blood in pregnancy direct endocrine IGF actions to particular tissues.

Specific Aims

1. To determine the effect of pregnancy and nutrition on the expression of IGF-I mRNA in livers of guinea pigs.

2. To determine the relationship between the relative abundance of hepatic IGF-I mRNA and circulating concentrations of IGF-I.

3. To identify the source of circulating IGF-I in pregnant guinea pigs.

4. To examine quantitative relationships between hepatic abundance of IGF-I mRNA and plasma concentrations of IGFs and IGF binding proteins with maternal, fetal and placental body composition.

CHAPTER 2

EXPRESSION OF IGF-I mRNA IN THE GUINEA PIG. DEVELOPMENT AND VALIDATION OF A REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION ENZYME LINKED IMMUNOSORBENT ASSAY

2.1 INTRODUCTION

Quantitative measurement of messenger RNA is controversial. In this study, two different nonisotopic methods were evaluated for their suitability for the quantitation of mRNA. Polymerase chain reaction (PCR) aims to amplify a defined section of DNA using repeat reactions with DNA polymerase simultaneously copying complementary DNA templates. Amplified cDNA products were evaluated by densitometric analysis of ethidium bromide stained cDNA on agarose gels or by digoxigenin ELISA where labelled digoxigenin-cDNA products were measured. RT-PCR was chosen as transcripts of low abundance can be amplified from relatively small amounts of RNA.

Quality of RNA extracted from tissues affects performance of reverse transcription of mRNA to first strand cDNA and subsequent PCR amplification as the product of both depends on the condition of the RNA template and the presence of contaminants, including DNase and RNase. Extraction of RNA must be performed under extremely clean RNase free conditions (Coleman, 1997). Integrity of RNA on an ethidium bromide stained agarose gel provides information with respect to RNase contamination by evaluation of ribosomal RNA degradation. Greater than 80% of RNA in an RNA extract from tissue will be ribosomal RNA (28S) and (18S) and will be electrophorectically separated into two distinct bands on an agarose gel (Jones, 1994). RNase activity degrades the 28S ribosomal band into many smaller bands that appear as a smear on the gel.

Complementary DNA was synthesised from a RNA template using the enzyme reverse transcriptase and random hexamer primers which produces cDNAs of variable lengths. Oligo (dT) primers are also commonly used for reverse transcription. They hybridise with the poly (A) tail found in most mRNA and lead to transcription from the 3' end producing full-length

cDNAs. Transcription of the gene of interest may be missed if its mRNA is lacking a poly (A) tail or may occur with variable efficiency if variable polyadenylation occurs.

For PCR of the initial cDNA reverse transcribed from mRNA, two oligonucleotide primers that span the target sequence are designed to be complementary to nucleotide sequences in opposing complementary DNA. The general method of PCR involves the amplification of cDNA by multiple cycles of heat denaturing allowing double stranded cDNA to be separated into single complementary strands, followed by cooling to anneal the oligonucleotide primers to complementary cDNA, followed by an extension step at an intermediary temperature where DNA polymerase elongates the primers and synthesises copies of the DNA. Each DNA copy then acts as template for the subsequent cycle. This amplification typically requires 20 to 30 repetitive cycles to produce sufficient DNA of interest to be detected, depending on the sensitivity of the detection system and the efficiency of amplification.

When using RT-PCR to quantify mRNA the quality of RNA extracted, efficiency of the reverse transcriptase and the efficiency of amplification of cDNA in the PCR reaction must be controlled. The amount of product produced is doubled after every cycle of PCR amplification only under ideal theoretical conditions which are rarely achieved in practice. Amplification efficiency experimentally is often much less than 100%. If efficiency of amplification varies by even a small amount between different specimens the effect on amplicon product yield is very large over 20 to 30 repetitive cycles. Amplification efficiency is influenced by reaction buffer components, concentration of nucleotide reactants, primer concentration, reaction times and temperatures, polymerase concentration and stability and cDNA contaminants.

Quantification of amplified PCR products is commonly based on the measurement of density of each cDNA product, expressed relative to a known standard or an internal control, after

agarose gel electrophoresis and ethidium bromide staining. The development of computer software and digital cameras has greatly improved the accuracy of measurement of these electrophoretic bands. However, sensitivity and linearity of response in these methods are not generally evaluated. Therefore different approaches have been taken for the quantitative evaluation of PCR amplified cDNA. Quantitative multiplex RT-PCR was developed so that the gene in question and an internal standard were co-amplified in simultaneous reactions and the amounts of each product can be compared. However this method has several disadvantages (Bustin, 2000).

For the purpose of this present study IGF-I mRNA was amplified by RT-PCR in a separate reaction to an internal reference gene β-actin. IGF-I mRNA gene expression was expressed relative to the abundance of β-actin mRNA in the same preparation of first strand cDNA. This approach enables amplification of both IGF-I and β-actin cDNA to be achieved without competition for reactants by different reactions and reduces nonspecific hybridisation that is often significant in multiplex assays. β-actin is ubiquitously expressed and is highly abundant in liver and adipose tissue. Expression patterns of many alternative genes used as a reference can vary due to developmental state and/or experimental treatment including use of anaesthetics (Thellin et al., 1999). Thus selection of reference genes must be based around specific tissues and cells and the study design. Ideally more than one reference gene with constitutive expression should be used to normalise the expression of the target mRNA. However the use of one gene has been widely accepted and β-actin is a common choice (Sturzenbaum and Kille, 2001;Thellin et al., 1999).

Two size classes of IGF-I mRNA transcripts have been reported in liver, bone and cartilage of guinea pigs by Northern blotting (Gosiewska et al., 1994). The abundance of IGF-I mRNA in liver was higher than in bone and cartilage. In rats and mice the liver is the major source of endocrine IGF-I, while in pigs and humans adipose tissue may be an alternative source of

endocrine IGF-I (Coleman et al., 1994;Moller et al., 1991). Therefore expression of IGF-I mRNA in guinea pig liver and adipose tissue was compared. Specific assays for guinea pig IGF-I mRNA and guinea pig β-actin mRNA were developed.

In order to quantitatively measure abundance of IGF-I mRNA in the guinea pig a suitable RT-PCR method was developed. In developing this method several steps were evaluated to be certain the gene of interest was amplified and that physiologically significant differences in expression of IGF-I mRNA were detectable. Amplified PCR products must be of expected size and identity of PCR products can be confirmed by nucleotide sequencing. Quantitative evaluation of amplified PCR products was performed by ethidium bromide staining of PCR products after agarose gel electrophoresis and by digoxigenin-ELISA. The efficiency of amplification, sensitivity, specificity, reproducibility and throughput were determined for both methods.

aluminium foil covered block was chilled in an esky containing ice and was used as a chopping board. A small piece of autoclaved aluminium foil pre chilled at -20°C for 1 hour was placed on the top of the chilled block. A piece of tissue of ~160 mg was excised and minced with ice-chilled scissors. Tissues were homogenised in 1 ml of **TRIZOL** per 100 mg of tissue (see below) using a chilled glass tissue grinder (Kontes Glass Company, Vineland, New Jersey). The minced tissue was placed into pre-weighed sterile tubes (Falcon, Becton Dickinson Labware, New Jersey) containing 1.6 ml of **TRIZOL** reagent. The tubes were then reweighed and the weight of tissue excised determined. The mixture was homogenized with 15-20 strokes of the chilled glass tissue grinder until a homogeneous pink suspension was obtained. The homogenate was decanted into a sterile 2 ml safe lock tube (cat no.0030 120.0941 Eppendorf- Netheler-Hinz-GmbH Germany tube) and held on ice until all samples were processed (between 2.5 to 4 h).

After use the tissue grinder was washed twice with 1M sodium hydroxide performing 10 strokes each wash, then two washes with 1% SDS in sterile water, two washes with 1% Triton X-100 in sterile water and finally washed with sterile water until all bubbles were removed. The cleaned grinder was returned to ice for re-use when required. This wash procedure was repeated after every tissue homogenisation to minimise contamination.

Safe lock tubes containing homogenates were centrifuged at 12 000 x g for 10 mins at 4°C (Eppendorf, 5415 C centrifuge) to pellet cellular debris. Five hundred µl of supernatants were pipetted to fresh safe lock tubes and incubated at room temperature (23°C) for 5 mins to permit dissociation of nucleoprotein complexes. Chloroform (0.32 ml) was added to each supernatant, which was then shaken vigorously by hand for 15 secs followed by incubation at room temperature for 3 mins. RNA remains in the upper aqueous phase while DNA is in the interphase and protein is in the lower organic phase. The three phases were separated by centrifugation for 15 mins at 12 000 x g at 4°C. Most of the colourless upper aqueous phase

containing the RNA (0.8 ml) was carefully removed and pipetted into fresh safe lock tubes carefully avoiding disturbance or removal of the interphase and lower organic phase. RNA was precipitated from this supernatant by mixing with 0.8 ml of isopropyl alcohol, incubation at room temperature for 10 mins, then centrifugation at 12 000 x g for 10 mins at $4^{o}C$. The resultant gel-like pellet was washed by re-suspension in 0.5 ml of 75% ethanol in sterile water. The RNA was pelleted by centrifugation at 7 500 g for 5 min at $4^{0}C$. The supernatant was aspirated and the pellet was air-dried for 15 mins at room temperature then dissolved in 50 µl of sterile water. The aqueous solution of RNA was divided into two aliquots. One was stored at $-80^{o}C$ and the second aliquot was stored at $-80^{o}C$ under 2.5 volumes of 100% ethanol and $1/25^{th}$ volume of 5M sodium chloride (Sambrook, 1989).

Concentration and integrity of RNA

The concentration and purity of RNA was determined from its UV absorbance spectrum (Beckman DU-50 Spectrophotometer Fullarton CA USA). Two µl of RNA was diluted with 198 µl of molecular grade water (Fluka, Chemika-Biochemika, Messerschmittstr, GmbH) and the absorbance spectrum from 260 nm to 300 nm was measured. Phenol contamination of samples was assessed by a shift in the peak absorbance from 260 nm to 270 nm. A ratio of absorbance at 260 nm to 280 nm of 1.6 or greater indicates RNA of acceptable purity (Davis, 1986). Protein contamination will be evident if the ratio is less than 1.6. An absorbance of 1 at 260 nm is equivalent to 40 µg/ml of RNA (Davis, 1986).

Total RNA concentration extracted was calculated by:

$$\text{Concentration of RNA } \mu g/\mu l = A_{260} \times 100 \times 40/1000$$

Agarose gel electrophoresis of RNA extracts followed by staining with ethidium bromide allowed the integrity of the RNA to be assessed by examination of the ribosomal RNA subunits 28S and 18S. RNA extracted from liver and fat were electrophoresed at 70 V for 1.5 hrs through a 1% (w/v) agarose gel in 0.5 x TBE buffer (0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA pH 8.0). The gel was then stained by soaking in 0.5 μg/ml of ethidium bromide for 30 mins, de-stained with water, transilluminated with UV light (Chromato-VUI Transilluminator TM-15, San Gabriel USA) and photographed with Kodak digital camera. Densitometric analysis was performed by Kodak dS Digital Science™, 1D Image Analysis Software Electrophoresis Documentation and Analysis System 120 (EDAS 120) (Kodak, Rochester, NY).

2.2.3 Reverse Transcription (RT)

Total RNA was reverse transcribed to cDNA using RNA-directed DNA polymerase (Expand™ reverse transcriptase, Cat No. 1785826 Boehringer Mannheim, Mannheim Germany). This is a Moloney murine leukaemia virus reverse transcriptase genetically engineered to remove RNase H activity. The enzyme was suitable for use with the PCR enzyme Taq polymerase, and incorporation of digoxigenin-labelled nucleotides (Bustin, 2000;Coleman, 1997).

There are three different forms of primers commonly used for cDNA synthesis prior to quantitation by PCR. These are oligo(dT), random sequence hexanucleotides and sequence specific primers. Random hexamers were used in the present study to produce a large population of cDNA fragments. Two μg of RNA extract in 20 μl of sterile water was mixed with 2 μl of random hexamer primers (100 μg/ml in sterile water, GeneWorks Pty. Ltd. Adelaide Aust. Cat rp-6), heated to 65^0C for 10 mins and then cooled on ice for 3 mins. A

"master mix" comprising of 8 µl of 5 x cDNA synthesis buffer (250 mM Tris-HCl, 200 mM KCl, 25 mM $MgCl_2$, 2.5% Tween-20, pH 8.3), 4 µl of 100 mM dithiothreitol (DTT) in water, 4 µl of Ultrapure dNTPs (10 mM each dATP, dCTP, dGTP, dTTP in water pH 7.5 (Amersham Pharmacia Biotech)) and 2 µl of Expand™ reverse transcriptase enzyme (100 units) was prepared. Eighteen µl of the "master mix" was added to the cooled 22 µl of RNA and random hexamer solution. The reaction mixture was incubated for 10 mins at 30^0C, to allow the random hexamers to anneal to the RNA. The mixture was heated for 45 mins at 42^0C allowing the reverse transcriptase enzyme to synthesise cDNA. RNA secondary structures that may hinder progression are minimised above 42^0C (Coleman, 1997;Lewin, 1987). Finally the enzyme was inactivated by denaturation at 95^0C for 2 mins. The solution was stored at -20^0C.

2.2.4 Primer design for Polymerase Chain Reaction (PCR) amplification of guinea pig IGF-I cDNA and β-actin cDNA

The design of primers affects specificity of hybridisation, size or length of cDNA amplified (amplicon) and efficiency of PCR amplification. Two primers are designed to be complimentary to the opposite complementary strands of the target DNA template and flank a sequence of interest. If they flank a genomic sequence that spans an intron it is possible to detect genomic DNA contamination of RNA preparations because genomic DNA amplification will give rise to a PCR product of greater length than that derived from cDNA.

Several guidelines are well established in the literature (Coyne, 1996;Mullis, 1994;Ruiz, 1997) and those guidelines used for designing IGF-I and β-actin primers are listed in Table 2.1. The primer length and G + C content are important in determining the melting temperatures (T_m) of the primer-cDNA hybrid. The annealing temperature chosen is usually

5°C below the melting temperature. However the annealing temperature affects specificity and amplification yield. In general the longer the primer and higher G +C content the higher the melting temperature. Primers are designed to have between 50 to 60% G+C bases and to be 18 to 28 nucleotides in length. Primers smaller than 18 nt are less representative of a unique DNA sequence and have a greater chance of hybridising to non-target DNA and hence lead to amplification of nonspecific cDNA products(Coleman, 1997;Coyne, 1996). Yields for amplicons greater than 2000 bp in length are generally very poor and amplicons less than 200 bp are difficult to resolve (from primer oligomers) on an agarose gel. Primers should be designed with less than 3 complementary bases at the 3' end to minimise formation of primer polymers. Primers should contain less than 3 G or C at the 3' end to avoid nonspecific hybridisation to cDNA, although including two has been suggested to anchor the primer to the recognition site. The concentrations of primers used in the present study were between 0.1-0.5 μM, which is reported to minimise mis-priming and accumulation of non-specific products and primer polymerisation (Coyne, 1996;Innis, 1990).

Table 2.1

Criteria for design and use of primers

Primer length	18-28 bp
G + C content	50-60%
T_m of primer pair	matched
	range 55° - 80°C
	$T_m = 2°C \times (A + T) + 4oC \times (C + G)$ *
	for primers 20 bp or less
Amplicon size	less than < 2000 bp
3' end matches	< 3
Adjacent homologous bases	< 7 bases
Runs of bases	< 3 bases
No of G or C at 3' end	<3
Concentration	0.1 -0.5 µM

T_m: melting temperature,*A, T, G, C, are the number of bases in the designed oligonucleotide.

References: (Coyne, 1996;Mullis, 1994;Ruiz, 1997)

Polymerase Chain Reaction Primer Pairs

Guinea pig IGF-I

Primers (Table 2. 2) were designed to hybridise to the sense and antisense partial cDNA sequence for guinea pig IGF-I DNA (GenBank X 52951) (Bell et al., 1990). PRIMER DESIGNER software package (Scientific and Educational Software, Stateline PA USA, Version 2) was used for designing primers following the criteria in Table 2.1. Figure 2.1 is a schematic representation of the guinea pig IGF-I partial gene structure. Reverse-phase HPLC quality (90 -97% purity) IGF-I primers were synthesised by SIGMA GENOSYS (Sigma-Aldrich, Castle Hill NSW

Table 2.2. Oligonucleotide primers designed for amplification of guinea pig cDNA by PCR

Name	Sequence	Predicted Amplicon Size	cDNA Nucleotides	GenBank Accession No.
gp IGF-I$_{310}$	F 5'-ACCTCTTCTACCTGGCCT-3'	236bp	310 to	X52951
gp IGF-I$_{545}$	R 5'-GCAGTACATCTCCAGCCT-3'		545	
r β-actin$_{1456}$	F 5'-TGTGATGGTGGGTATGGGTC-3'	372bp	1456 to	V01217
r β-actin$_{2291}$	R 5'-TAGATGGGCACAGTGTGGGT-3'		2291	

*F = Forward primer, R = Reverse primer, * denotes expected amplicon size using the rat β -actin gene sequence*
subscript numbers denotes the nucleotide where the forward primer starts or where the reverse primer ends

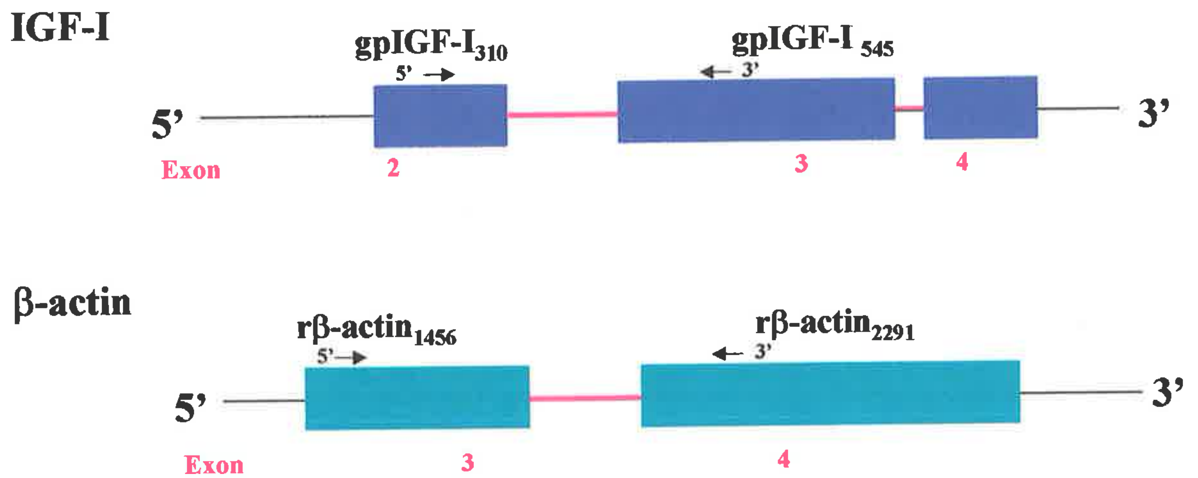


Figure 2.1 Schematic representation of the partial guinea pig IGF-I gene and rat β-actin gene.

From the 5' flanking region the coding regions of exons (blue) for guine pig IGF-I and (green) for rat β-actin. Introns are red lines and flanking regions are thin black line. Introns for gpIGF-I gene were based on the human IGF-I gene. The black arrows indicate primer binding sites. IGF-I (Genebank accession number X52951), β-actin (Genebank accession number V01217). Subscripts represent the cDNA nucleotide where the forward primer starts and the reverse primer ends.

β-Actin

Measurement of the abundance of β-actin mRNA was used as a reference for IGF-I mRNA abundance in the same first strand cDNA. Primers for the amplification of guinea pig β-actin cDNA were designed using the rat DNA sequence (GenBank V01217 J00691). The β-actin sequence available for the guinea pig was only 138 bp, which is too short to design useful primers for PCR amplification. Rat β-actin cDNA sequence was found to have 88% homology with guinea pig β-actin cDNA (partial cDNA sequence Genbank accession No AF193571) after analysis by BLASTIN 2.1.2 software available at http://www.ncbi.nlm.nih.gov:80/blast/b12seq/.

2.2.5 Polymerase Chain Reaction

PCR amplification of 5 ul of cDNA preparations (section 2.2.3) was performed using the *Taq* DNA Polymerase Kit (Fisher Biotech International, WA) in PCR buffer (67 mM Tris-HCl, pH 8.8 [at 25^{0}C], 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml gelatin), also containing 2 mM $MgCl_2$, 200 uM of ultrapure dNTPs [dATP, dCTP, dGTP, dTTP], (Pharmacia Biotech), 0.5 µM of primers IGF-I_{310} and IGF-I_{545} or β-$actin_{1456}$ and β-$actin_{2291}$ plus 0.02 U/ul of Taq DNA polymerase, in a final volume of 50 µl.

IGF-I and β-actin cDNA fragments were amplified by PCR in different reactions performed under the same conditions. PCR was performed in a thermal cycler (PCR Express, Hybaid Ltd., UK). An initial denaturation at 94°C was performed for 3 mins followed by repeated cycles of denaturation at 94°C for 15 sec, annealing at 58°C for 15 sec and extension at 72°C for 45 sec. A terminal extension at 72°C was performed for 3 mins

The amount of DNA in PCR products was determined from spectrophotometric analysis at 260nm where 50μg/ml of DNA = 1 absorbance unit.

2.2.6 Calculation of the molecular size of PCR products

The IGF-I DNA PCR product was diluted 1:5, 1:10 and 1:20 and 4 replicates of 7 ul of each solution and duplicates (0.25 μg) of a DNA molecular size standard pUC19 DNA digested with *HpaII* (GeneWorks, Adelaide, Australia) were electrophoresed at 90V for 45 mins through a 2% (w/v) agarose gel in 0.5 x TBE buffer. The gel was stained and analysed as described in section 2.2.2. PCR product abundance as quantitatively determined by measuring the density in pixels (net intensity) of selected bands, expressed as a % of the net intensity of the 404 bp fragment of known mass of the pUC19 DNA/*Hpa II* standard. The results presented are the mean net intensity of the replicates of the PCR products electrophoresed. The Kodak EDAS 120 (section 2.2.2) was used to measure the mobilities of the pUC19DNA/*HpaII* fragments of known size and the IGF-I and β-actin cDNA products whose size was to be measured. The size of the DNA fragment was calculated by plotting log DNA size in nucleotides for the duplicate pUC19/*HpaII* standards (y) versus distance migrated, (x). A linear regression was performed (Sigma Stat) to generate a linear equation. The equation is of the format $y = mx + b$, therefore the equation of this plot is Log DNA size = slope x mobility + y-intercept. The distance migrated of each of the replicates IGF-I and β-actin DNA was measured and the size calculated by use of the equation.

2.2.7 Sequencing of Polymerase Chain Reaction products

Products amplified with IGF-$I_{310/545}$ or β-$actin_{1456/2291}$ primers were purified with the QIAquick PCR Purification Kit (QIAGEN, Melbourne Vic) and sequenced using the ABI PRISM TM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer,

Branchburg NJ USA). The following reagents were added to PCR tubes; 8 μl of ready reaction mix (BigDye™ primers, dNTPs, AmpliTaq® FS enzyme, $MgCl_2$ and reaction buffer), 24 ng of purified PCR product DNA, 4 μl of primer (3.2 pmol), to a final reaction volume of 20 μl with sterile water. Each primer IGF-I_{310}, IGF-I_{545}, β-$actin_{1456}$ and β-$actin_{2291}$ was added to different aliquots of purified PCR products, thus, producing 4 products for nucleotide sequencing. Amplification of cDNA was performed by heating the reaction mixture to 96°C followed by 25 cycles at 96°C for 30 secs, 50°C for 15 secs and 60°C for 4 mins. Amplified products labelled with terminator dye were placed directly on ice on completion. The cDNA in 20 μl of the reaction mix was precipitated by mixing with 2 μl of 3M sodium acetate pH 5.4 and 50 μl of 95% ethanol and incubated on ice for 10 mins followed by centrifugation at 12 000 x g for 30 mins. The resultant pellet was resuspended in 250 μl of 70% ethanol. The cDNA was re-pelleted by centrifugation at 12 000 x g for 20 mins at 4°C. The pellets were partially air-dried. Sequencing of the pellets was performed by the sequencing centre at the Institute of Medical and Veterinary Science (IMVS, Frome Rd Adelaide SA).

2.2.8 Determination of IGF-I and β-actin efficiency of amplification

To determine the efficiency of amplification of IGF-I and β-actin a large stock of liver cDNA was prepared by reverse transcription of guinea pig liver RNA (section 2.2.3) for this purpose. Reverse transcription of 15 replicates of 2 μg of liver RNA extract was performed, at the end of which all cDNA products were pooled making a large stock of guinea pig liver cDNA. The stock was divided into duplicate aliquots designated as $cDNA_1$ and $cDNA_2$. A PCR master mix with IGF-I_{310}/IGF-I_{545} primers, as described in 2.2.6, was prepared and added to 22 replicates of either gp liver $cDNA_1$ or gp liver $cDNA_2$ to a final volume of 50 μl. A second master mix with β-actin primer pairs was added to another 22 replicates of gp liver $cDNA_1$ to a final volume of 50 μl. They were then subjected to PCR using the same cycling parameters

as described in section 2.2.6, excluding the final extension cycle of 3 min at 72°C. Duplicates were removed from the thermal cycler every 2nd cycle from 20-36 cycles at the end of the 45 sec, 72°C extension step and placed directly on ice. The thermal cycler was not stopped at any time and only opened and quickly closed at the end of the 45 sec extension step. PCR products were then prepared for agarose electrophoresis by mixing 14 μl of each with 4 μl of 6x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in water (Sambrook, 1989). Five μl of solution was placed in each well in the electrophoresis gel, which was equivalent to 3.8 μl of PCR product. A standard pUC19 DNA/*Hpa II* was also loaded into a well on every gel (0.25 μg/5 μl, 2.5 μl of a 500 ng/μl stock was diluted with 5 ul of loading buffer and 17.5 μl of water). Electrophoresis was performed as described in section 2.2.2.

Calculation of efficiency of amplification

The theoretical maximum efficiency of PCR is 100% in which the amount of PCR product doubles every cycle. Product formation in PCR follows the general equation $y = ab^x$, where y = amount of product, a = amount of initial template, x = number of cycles, b = amplification factor ($1 \leq b \leq 2$), where b = 1 + E, and E is the PCR efficiency. The efficiency of amplification E can be calculated by linear regression of the log transformation of the equation;

$$y = a(1 + E)^x$$

$$\log y = \log [a(1 + E)^x]$$

$$\log y = \log a + x\log (1 + E)$$

Linear regression of a plot of log(amount of PCR product) versus number of cycles has an intercept of log (amount of initial template) on the y axis and a slope of log (1 + E). The antilog of this slope is equal to 1 + E.

Precision and reproducibility

Precision and reproducibility of the amplification of PCR products measured by densitometric analysis within and between electrophoretic gels was evaluated. The within assay variability was determined by amplifying $cDNA_1$ with IGF-I primers for 24 cycles, diluting 1:5, 1:10 or 1:20 with water and analysing each in quadruplicate on a single gel. Quadruplicates of each solution (7 µl) were applied to a 2% agarose gel and electrophoresed as described in section 2.2.2. The between-assay variability was determined by using duplicates of $cDNA_1$ and $cDNA_2$. These cDNA duplicates were amplified with $IGF\text{-}I_{310}/IGF\text{-}I_{545}$ primers under the same amplification conditions described in section 2.2.5. Duplicate cDNAs were amplified for 22 to 26 cycles inclusive in the same thermal cycling batch. PCR product was analysed in duplicate on separate gels (Figure 2) resulting in quadruplicates for each cDNA at each of the cycle numbers listed above. PCR products (14 µl) were diluted with 4µl of electrophoresis loading buffer and 5µl was loaded per well and subjected to electrophoresis as in section 2.2.2. The intensity of the ethidium bromide stained band was determined using the Kodak EDAS 120 system calibrated using the density of the 404 bp pUC19/*Hpa II* DNA standard (0.25 µg) loaded per well. The pUC19/*Hpa II* DNA (0.25µg/5µl) standard was loaded in at least two wells per gel and the average used for mass calibration.

2.2.9. Enzyme Linked Immunosorbent Assay of Digoxigenin-labelled cDNA

Roche Diagnostics GmbH, Mannheim (Mannheim, Germany) have developed a method for the quantitative analysis of PCR products which involves the incorporation of a digoxigenin-labelled nucleotide, digoxigenin-11-dUTP, by including it with the nucleotide reactants in the PCR reaction. The Dig-labelled cDNA product is then hybridised to a biotinylated complementary oligonucleotide. The resultant biotinylated hybrid is bound to a streptavidin coated microtitre plate. The amount of digoxigenin captured to the microtitre plate is detected by monitoring the formation of a coloured product at absorbance 405 nm following the addition of a peroxidase-conjugated anti-digoxigenin antibody and ABTS (2,2'-Azino-di-[3-

ethylbenzthiazoline sulfonate] diammonium salt) substrate. This provides a highly sensitive and specific DNA ELISA system.

Biotinylated capture oligonucleotides

Biotinylated oligonucleotides, gp IGF-I and gp β-actin, were designed to be complementary to the sequences obtained from section 2.2.7 and were synthesised and desalted by GeneWorks and Genosys respectively. The 5'-biotinylated oligonucleotides are described in Table 2.3.

Table 2.3 Biotinylated oligonucleotides that bind streptavidin coated ELISA plates for the immobilisation of Dig-labelled PCR products

Name	Sequence of 5' biotinylated oligonucleotide	PCR product nucleotide	Length bp	%GC
gp IGF-I	5'-TCCTCACCAGCTCGGCCACA-3'	341 - 359	21	65
gp β-actin	5'-TCTGGCACCACACCTTCTAC-3'	31 - 49	21	55

Digoxigenin labelling of IGF-I and β-actin PCR products

Dig-labelling (Figure 2.2) of cDNA was performed using PCR conditions as described in section 2.2.5. The effect of the concentration of Dig-dNTPs (Boehringer Mannheim GmbH), amount of template cDNA and amount of PCR product added to the ELISA was determined. Five µl of guinea pig liver cDNA, equivalent to 0.25 µg of RNA, and 2.5 µl equivalent to 0.125 µg of RNA, were each aliquoted into 6 replicate PCR tubes containing variable concentrations of Dig-dNTP mixture (40, 80, 100 and 200 µM). These aliquots were subjected to PCR, as described in 2.2.5 for 30 cycles, placed on ice then frozen. Quantitation of 5 µl or 10 µl of Dig-labelled PCR products was performed using the Dig-ELISA (Figure 2.3).

Digoxigenin-labelled cDNA Enzyme Linked Immunosorbent Assay

Dig-labelled PCR products were thawed on ice and 10 µl diluted to a final volume of 250 µl with ELISA buffer (0.05M Phosphate buffer, pH 7.5) containing the appropriate biotinylated oligonucleotide ("capture probe") at 50 ng/ml. The mixtures were heat denatured at 94°C for 10 mins then incubated at 60°C for 10 mins to allow hybridisation to occur between the biotinylated capture oligonucleotide and the Dig-labelled PCR product followed by incubation at room temperature for 10 mins. The solution of hybridised biotinylated oligonucleotide and Dig-labelled PCR product were mixed and centrifuged at 10 000 rpm for 30 secs and 200 µl was added to a streptavidin-coated microtiter plate well (cat no 1734776, Boehringer Mannheim, GmbH, Germany). The plate was covered with transparent adhesive film and incubated for 3 hrs at 37°C shaking at 160 rpm in an orbital incubator (Orbital Mixer Incubator, Ratek Instruments). The plate was washed 3 times with wash buffer (Boehringer Mannheim, Germany). The anti-digoxigenin-polyperoxidase conjugate antibody (Fab fragments), 200 µl of 40 U/l (Boehringer Mannheim), diluted in Conjugate Buffer (100 mM Na-PO_4, 50 mM NaCl, 0.5 mM EDTA, 0.1% Tween^{R}20, 1% casein, pH 7.5) was added and incubated for 30 mins at 37°C shaking at 160 rpm. The plate was washed a further 3 times with wash buffer. The substrate ABTS (0.5 mg/ml) was made by dissolving one tablet of ABTS^R (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt) in 10 ml of a 1/10 aqueous dilution of ABTS^R buffer (sodium perborate, citric acid and disodium hydrogen phosphate) in the dark. Two hundred µl of the ABTS solution was added per well and the plate was wrapped in foil, as the substrate is light sensitive. The reaction was allowed to proceed for 15-30 mins at 37°C shaking at 160 rpm. The spectrophotometric absorbance was measured at 405 nm (Microplate EL 310 Autoreader, Bio-Tek Instruments, USA).

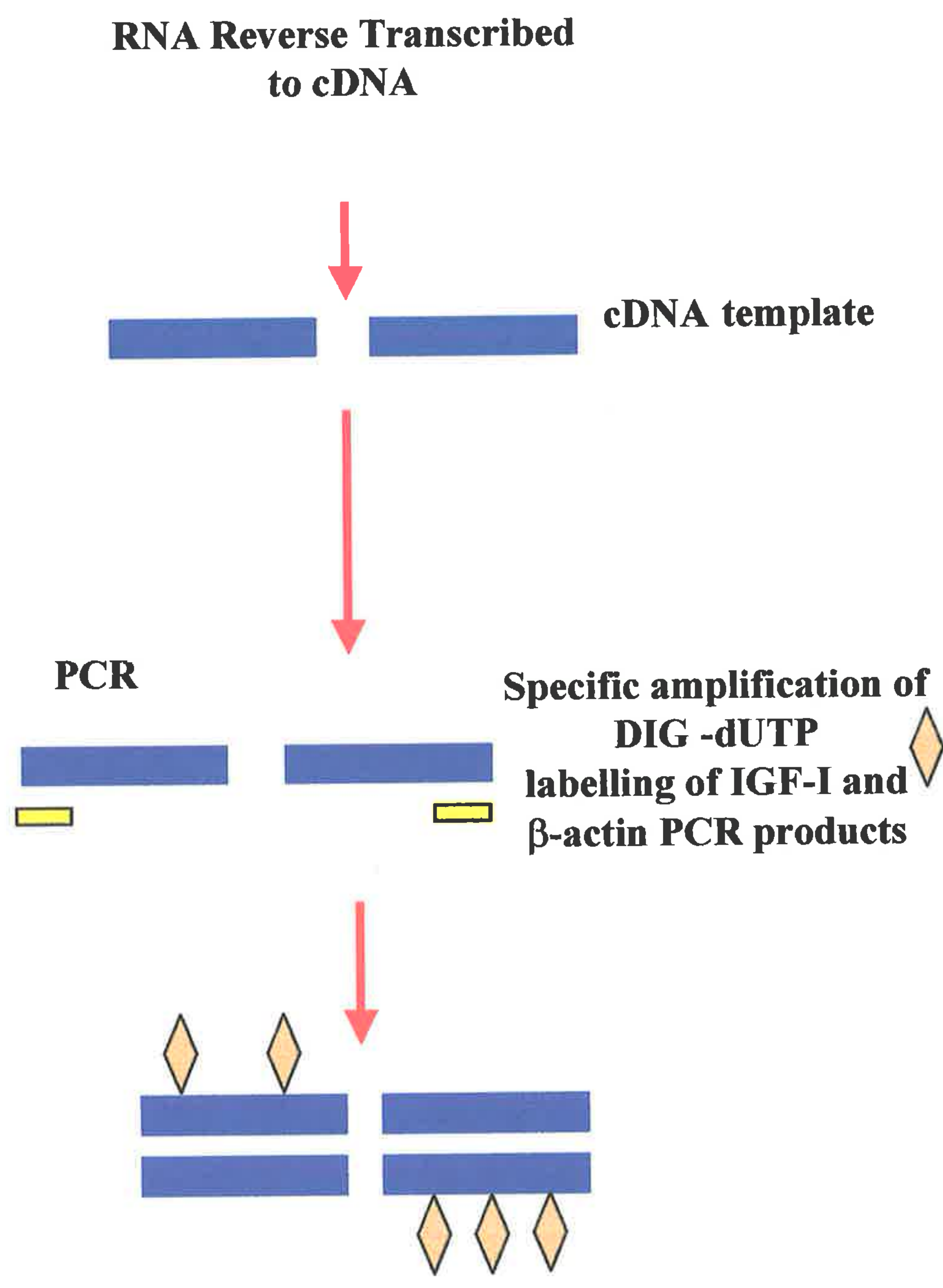


Figure 2.2 Schematic diagram of digoxigenin labelling of amplified PCR products

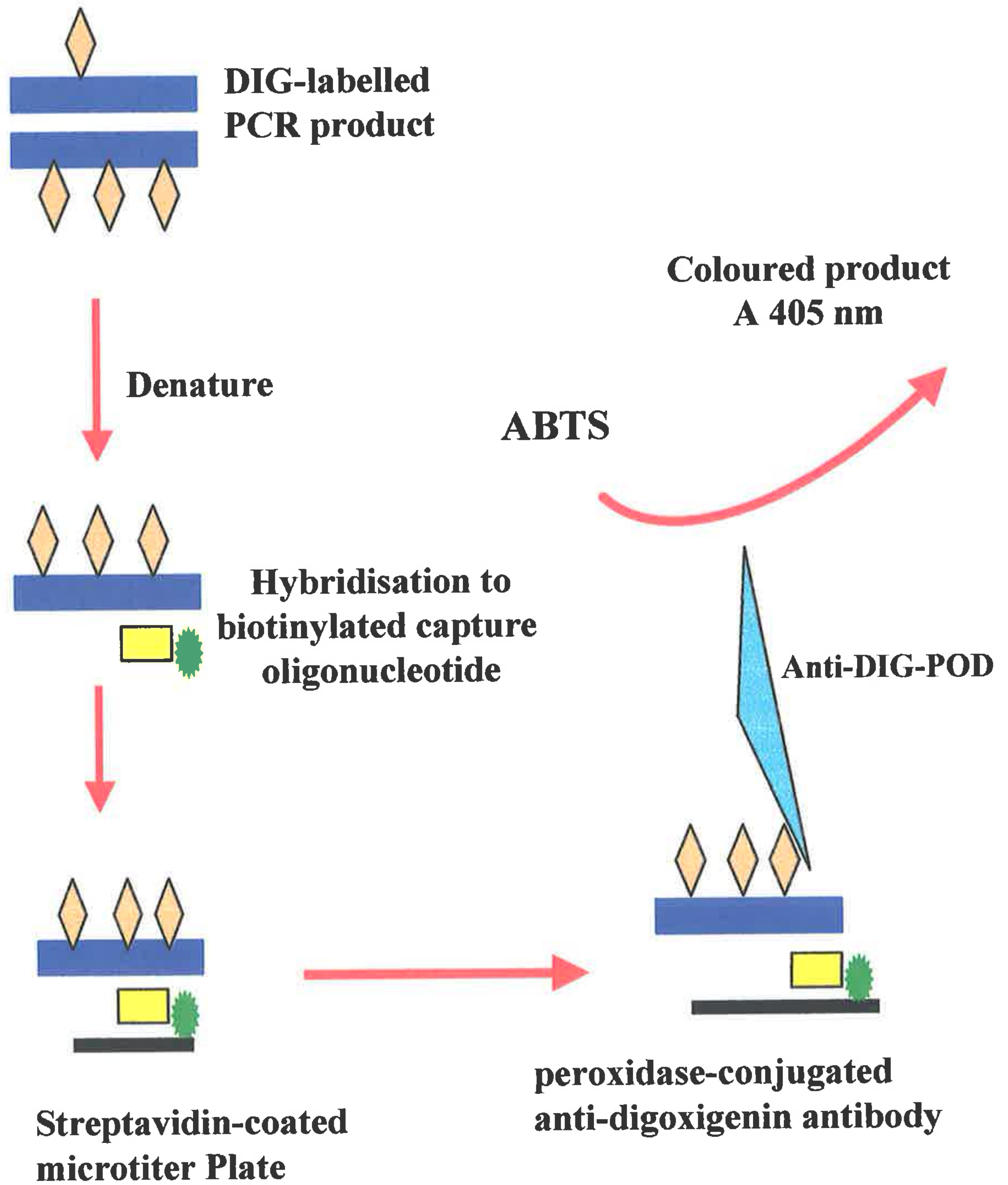


Figure 2.3 Schematic of PCR ELISA procedure for the quantitation of Dig labelled PCR products

Efficiency of PCR amplification of Dig-labelled IGF-I and β-actin

The efficiency of amplification of Dig-IGF-I cDNA was determined as described in section 2.2.8. except that a Dig-labelled dNTP solution replaced the unlabelled dNTP solution in the master mix. Dig-dNTP solution was used at a final concentration of 100 μM. A solution sufficient for 22 Dig-IGF-I PCR reactions was prepared. Sterile water (2.5 μl) was substituted for cDNA in replicate reactions as a negative control or PCR blank. Sixteen replicates of guinea pig liver cDNA (2.5 μl) were aliquoted into flat top PCR tubes with 47.5 μl of the Dig-IGF-I PCR master mix. PCR was performed by heating at 94°C for 3 mins, followed by 36 cycles of 94°C for 15 secs, 58°C for 15 secs and 72°C for 45 secs. Replicates were removed every second cycle at the end of the extension phase (72°C) from cycle 18 to 36. The Dig-IGF-I cDNA products were measured by Dig-IGF-I DNA ELISA as described above. Amplification efficiency of Dig-β-actin cDNA was determined as described above except a Dig-β-actin PCR solution was prepared sufficient for 16 reactions and replicates were removed every second cycle from 16 to 26 and the Dig-β-actin cDNA products were measured by Dig-β-actin DNA ELISA.

Precision and reproducibility

The between PCR batch coefficient of variation was measured in one ELISA. This was determined by amplification in triplicate of guinea pig liver $cDNA_1$ and $cDNA_2$ (2.5 μl). Replicates $cDNA_1$ and $cDNA_2$ were Dig-labelled in three independent PCRs. Ten μl of each Dig-IGF-I $cDNA_1$ or Dig-IGF-I $cDNA_2$ product was assayed in duplicate in ELISA, therefore for each Dig-IGF-I $cDNA_1$ and $cDNA_2$ 6 replicate wells were measured (section 2.2.9) for each PCR.

Precision of the Dig-labelled PCR ELISA was evaluated. The within PCR variability was determined using triplicates of a guinea pig liver cDNA amplified for 24 cycles in one IGF-I PCR. The Dig-IGF-I cDNA products (n = 3) are diluted 1:25 and assayed in duplicate (200 μl) in the ELISA (n =6). The mean and standard deviation was used to calculate the coefficient of variation %CV = (standard deviation/mean) x 100. The between assay variability was determined by using Dig-labelled IGF-I cDNA as a calibration standard (see below for its preparation). The coefficient of variation was evaluated from three independent assays using the absorbance measurement of 2 μl of the Dig-labelled IGF-I cDNA standard.

Preparation and quantitation of a Dig-labelled guinea pig liver IGF-I and β-actin PCR standard

Standards were prepared to calibrate measurement of Dig-DNA. Guinea pig liver RNA was reverse transcribed, as per 2.2.3, to produce a stock of gp liver cDNA, which was employed for the preparation of a stock of Dig-IGF-I DNA and a stock of Dig-β-actin DNA as follows. Each stock cDNA (10 μl) was aliquoted into 36 tubes. A master mix containing gp IGF-I primers and Dig-labelled dNTP was added to 18 of the tubes up to a final volume of 50 μl and the remaining 18 aliquots were made up to 50 μl with a master mix containing gp β-actin primers and Dig-labelled dNTP. Negative controls were included for both primer pairs in which molecular grade water (10 μl) replaced cDNA for a PCR control as well as aliquots (10 μl) in which molecular grade water has been substituted for RNA in the RT reaction (negative control for RT). PCR was performed by heating to 94°C for 3 mins, followed by 36 cycles of 94°C for 15 secs, 58°C for 15 secs and 72°C for 45 secs and a final extension at 72°C for 2 mins. The resultant Dig-cDNA products for each primer pair were pooled and re-aliquoted into 50 μl lots for storage. Each Dig-cDNA standard was quantified by agarose gel electrophoresis (section 2.2.8). Each amplified product was serially diluted 1:5, 1:10, 1:20 and 7 μl was loaded in quadruplicate onto a 2% agarose gel that was subjected to

electrophoresis. The gel was stained by ethidium bromide and densitometric analysis performed as described in section 2.2.2, and the amount of product was determined by comparison with the intensity produced by the 404 bp *Hpa II* fragment of the pUC19 standard (section 2.2.6).

2.3 RESULTS

2.3.1 RNA Isolation

Total RNA was extracted from guinea liver with a yield ranging from 1.4 to 1.95 μg of RNA per mg of wet tissue weight. Total RNA was extracted from guinea pig parametrial fat, interscapular fat and retroperitoneal fat with yields ranging from 0.9 to 3.6 μg/mg, 0.6 to 1.1 μg/mg, and 1.9 to 2 μg/mg respectively. RNA extracts examined in ethidium bromide stained agarose gels showed two distinct bands, consistent with the 28S and 18S ribosomal RNA subunits at 4.5 and 1.9 kb (Figure 2.4). Very little smearing was observed migrating ahead of the 28S rRNA, indicating very little RNase activity. The ratio of 28S to 18S, assessed by densitometric scanning was 1.5 ± 0.08. The ratio of absorbance at A_{260} and A_{280} for RNA extracted from liver was greater than 1.8 indicating that there was minimal contamination by proteins, DNA or phenol.

2.3.2 Amplification of IGF-I and β-actin cDNA fragments

The reverse transcription products from guinea pig liver RNA subjected to PCR with primer pair gp IGF-$I_{310/545}$ amplified a single product (Figure 2.5 A). Dilution of the amplified products from the IGF-I PCR showed that the intensity of ethidium bromide staining was reduced with increasing dilution. Negative controls containing water (RT and PCR) amplified with primer pair gp IGF-$I_{310/545}$ resulted in no product formation. The size of the IGF-I DNA fragment was calculated by graphing Log pUC19/*Hpa II* DNA fragment size versus distance migrated in an agarose gel (Figure 2.5 B). Linear regression analysis was applied to the duplicate pUC19 DNA /*Hpa II* standard data which had a linear relationship of Log size DNA

fragment = -7.191×10^{-3} * (mobility) + 2.96. The mobility of each product was used to calculate the size of the fragment and the mean ± sem. Guinea pig IGF-I cDNA was found to be 243.2 ± 2.5 bp compared to its predicted size of 236 bp. Amplification of guinea pig β-actin cDNA, produced fragments of 383 ± 12.2 bp when calculated from the linear relationship of Log size DNA fragment = -1.692 x 10-3 * (mobility) + 2.295, compared to its predicted size of 372 bp from the rat β-actin gene sequence.

Liver

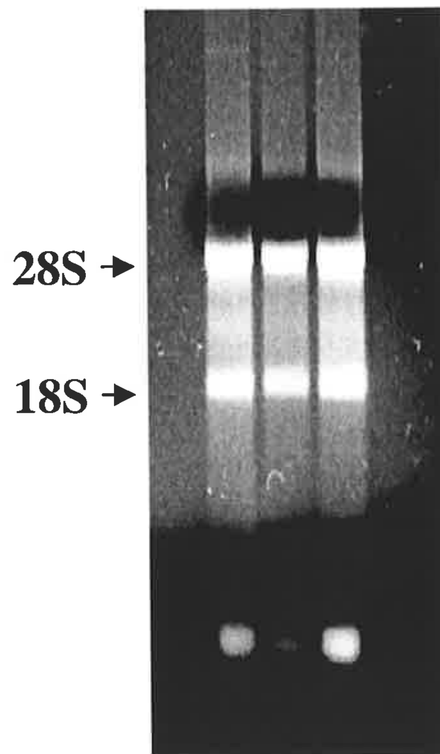
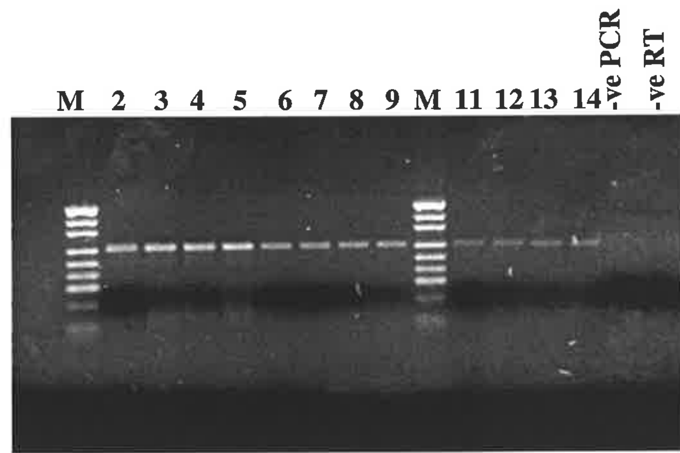


Figure 2.4 Agarose gel electrophoresis of total RNA

Agarose gel electrophoresis of 1.7 μg of liver RNA from 60 day pregnant guinea pigs after staining with ethidium bromide.

A

LIVER



B

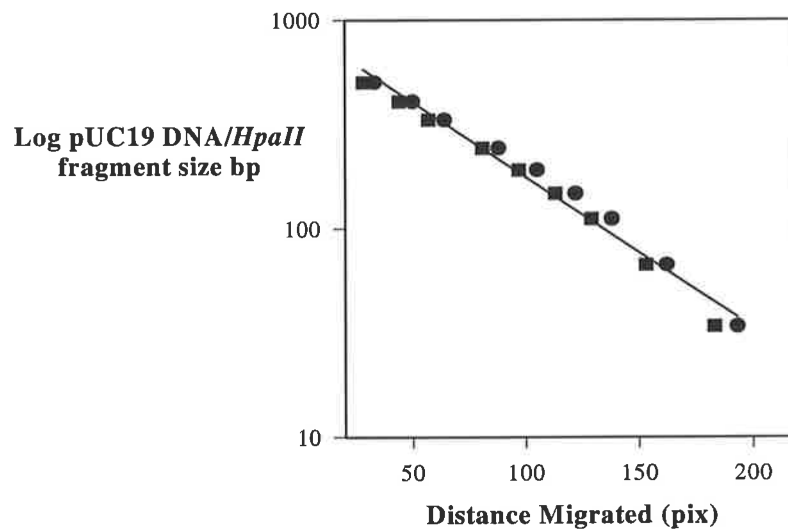


Figure 2.5 Agarose gel electrophoresis of RT-PCR products

(**A**) RNA from guinea pig liver was reverse transcribed and PCR performed using primers gp IGF-$I_{310/545}$. IGF-I cDNA was diluted 1:5 (Lanes 2 to 5), 1:10 (lanes 6 to 9) and 1:20 (lanes 11 to 14) and subjected to agarose electrophoresis. Negative PCR contains IGF-I primer pair, sterile water and PCR reaction solution. Negative RT contains IGF-I primer pair, water (substituted for RNA in RT reaction mixture) and PCR reaction solution. M = 0.25 μg of pUC19 DNA/*Hpa II* standard (range 111 - 501 bp fragments). (**B**) The size of the band was estimated to be 243.2 ± 2.5 bp (mean ± sem) by comparing the distance migrated by the amplicon against the migration of the DNA markers.

A single cDNA product was amplified from parametrial fat, interscapular fat and retroperitoneal fat with gpIGF-I primer pair and rβ-actin primer pair (Figure 2.6). The size of the IGF-I product was estimated as described above and was 241 $\pm$ 2.2 bp with a predicted size of 236 bp. The reference gene β-actin was estimated as 393 $\pm$ 3 bp with a predicted size of 372 bp. IGF-I was expressed in parametrial fat, interscapular fat and retroperitoneal fat as shown on agarose gel electrophoresis of RT-PCR products. Amplified IGF-I PCR products from fat tissues were undiluted, but liver IGF-I PCR products were diluted 1:10 before being subjected to agarose gel electrophoresis. Qualitative estimation of the ethidium bromide stained PCR products, subjected to agarose gel electrophoresis, suggests IGF-I mRNA was more abundantly expressed in the liver than any of the fat depots analysed.

2.3.3 Sequence of amplified PCR products

The fragments amplified from guinea pig liver cDNA by gpIGF-$I_{310/545}$ and rat β-actin primers were purified for sequencing. Sequence data was obtained for 171 bases out of the possible 243 for gpIGF-I (gpIGF-$I_{375-545}$) and 295 bases were sequenced for gp β-actin. The sequences were analysed by the BLASTIN 2.1.2 software (see section 2.2.4). The cDNA product amplified by gpIGF-$I_{310/545}$ aligned with 171 bases between nucleotides 375-545 and had a 100% identity to gpIGF-I mRNA (Bell et al., 1990) (Figure 2.7). A 295 base product sequence from gp liver cDNA, amplified using rat β-actin primers, aligned with the partial guinea pig heart β-actin sequence

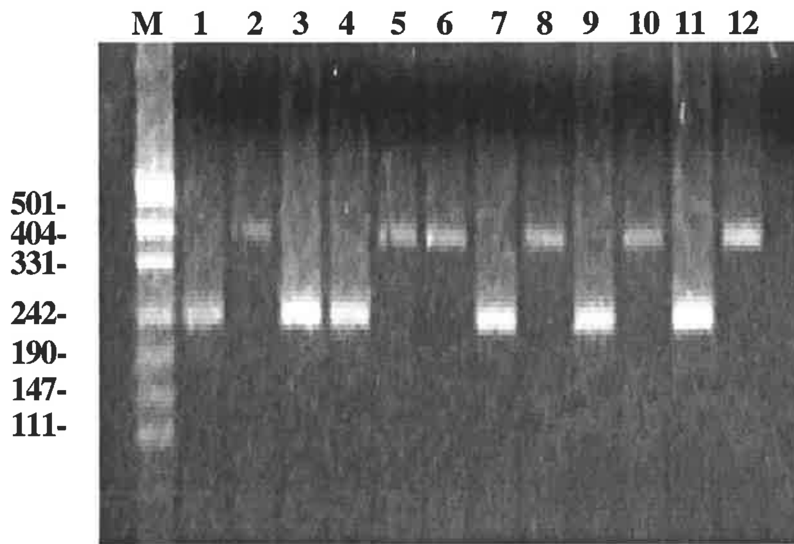


Figure 2.6 Agarose gel electrophoresis of adipose fat RT-PCR products

RNA from guinea pig parametrial, interscapular, retroperitoneal fat was reverse transcribed and PCR performed using primers gp IGF-$I_{310/545}$ or r β-$actin_{1456/2291}$. M = 0.25 μg of pUC19 DNA/*Hpa II* standard (range 111 - 501 bp fragments).

IGF-I cDNA products for parametrial fat shown in lanes 3 and 7, interscapular fat are shown in lanes 1, 4, and 11 and retroperitoneal fat is shown in lane 9.

β-actin cDNA products for parametrial fat are in lane 5 and 8, interscapular fat are in lanes 2, 6, and 12 and retroperitoneal fat is in lane 10.

The size of the IGF-I cDNA product was estimated to be 241 ± 2.2 bp (mean ± sem) and the β-actin cDNA product was 393 ± 3 bp by comparing the distance migrated by the amplicons against the migration of the DNA markers.

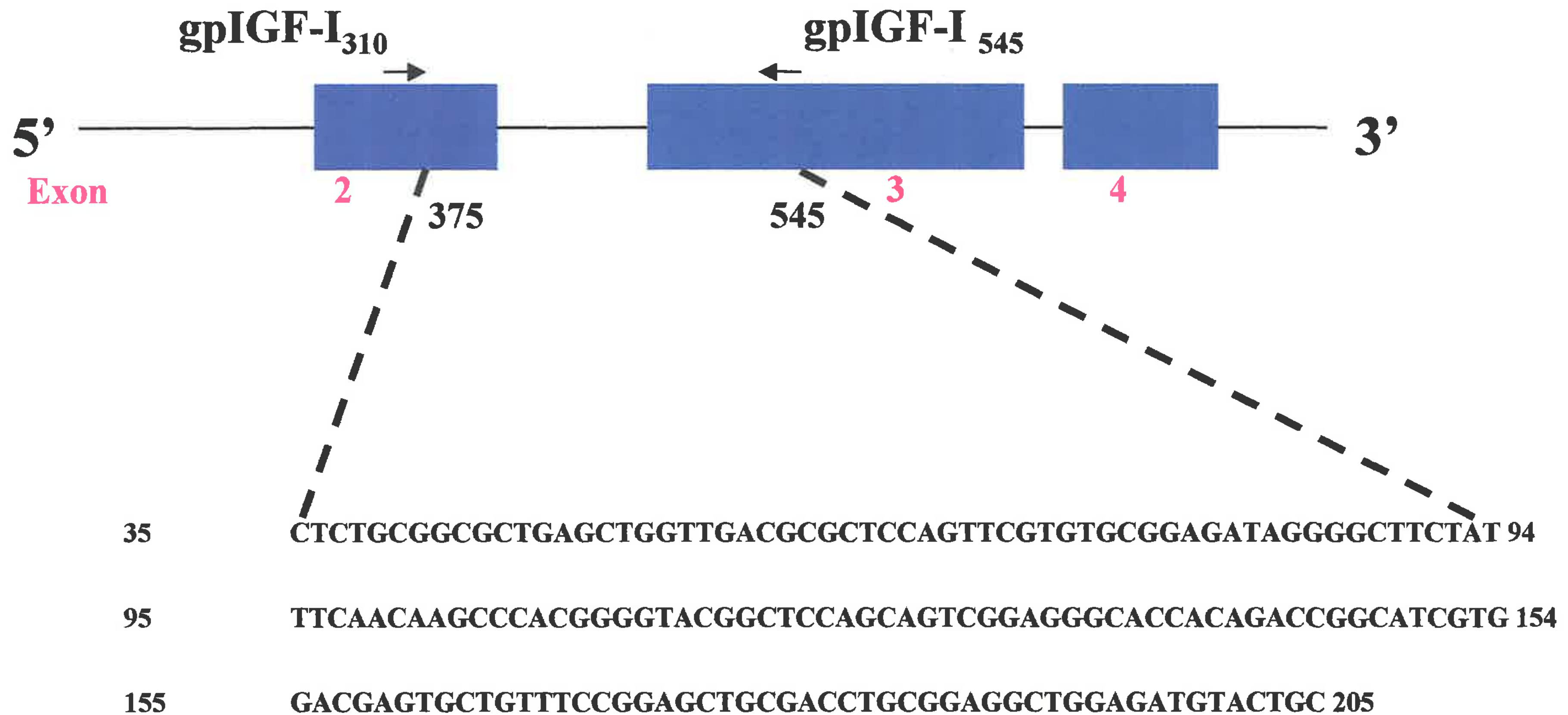


Figure 2.7 Proposed organisation of the guinea pig IGF-I cDNA and the partial nucleotide sequence of the amplified fragment using gpIGF-I$_{310/545}$ primers
The hatched lines are aligned with 375bp to 545bp of the guinea pig IGF-I cDNA sequence (Bell, 1990). The amplified product was identical to the guinea pig cDNA and extends from exon 2 into exon 3.

(GenBank AF 193571) at nucleotides 130 to 265 with 86% homology in this region. The amplified product of 295 bases was also aligned with the rat gene encoding cytoplasmic beta-actin (GenBank Vo1217). The sequence aligned at nucleotides 1473-1697 and 2155-2224 and was 85% and 91% homologous respectively to rat cytoplasmic β-actin (Figure 2.8).

2.3.4 Efficiency of amplification of guinea pig IGF-I cDNA and β-actin cDNA estimated by densitometric analysis.

RT-PCR of guinea pig liver RNA was performed and the efficiency of amplification calculated from the densitometric analysis of the PCR products after agarose gel electrophoresis and ethidium bromide staining. Guinea pig liver RNA was reverse transcribed in duplicate and then subjected to PCR either in duplicate or quadruplicate to assess the error contributed by RT, PCR and densitometric analysis in the quantitation of mRNA. Amplification of IGF-I and β-actin by PCR produced single products whose migration was intermediate of the 242 and 331 bp and the 404 and 501 bp markers respectively, which was consistent with the expected sizes. The efficiency of amplification (E) as calculated by the equation $y = ab^x$ (section 2.2.8), was 26% for IGF-I between cycles 20 to 24 inclusive, (Figure 2.9) and was 34.5% for β-actin between cycles 22 to 26 inclusive (Figure 2.10).

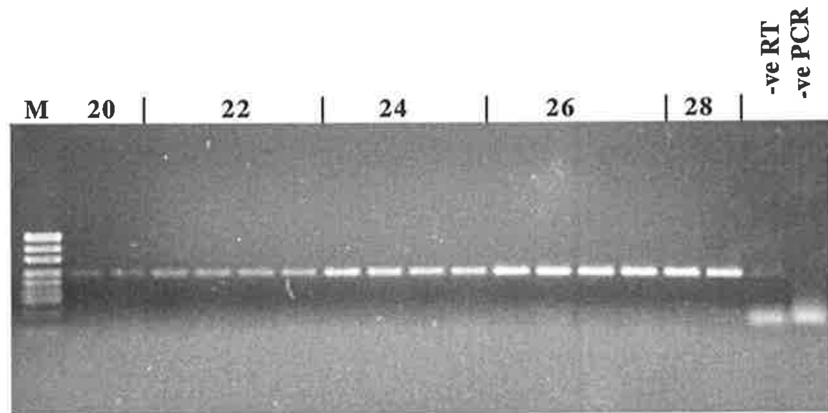
Precision

The coefficient of variation associated with estimation of the density of IGF-I PCR products the amplicons on agarose gels ranged from 1.9 % at cycle 28 to 20.5 % at cycle 20 (Figure 2.11). This indicates that densitometric measurement at lower numbers of amplification cycles is more variable. The within-assay variability was determined using the same cDNA which was amplified for 24 cycles, diluted 1:5, 1:10, 1:20 and analysed in quadruplicate on a single gel. Within-assay variability was found to range from 3% to 12%. The between gel

Figure 2.8. Sequence alignment of rat cytoplasmic, guinea pig liver and heart β-actin.

The invariant nucleotides in the guinea pig liver cDNA (coloured red) compared to the rat β-actin sequence. Comparison of guinea pig liver and guinea pig heart β-actin cDNA show that 14% of the nucleotides differ and these are shown in green.

RAT	1473	GTCAGAAGGACTCCTACGTGGGCGACGAGGCCCAGAGCAAGAGAGGCATCCTGACCCTGA	1532
β-actin cDNA product	346	GTCAGAAAGATTCCTACGTGGGCGACGAGGCCCAGAGCAAGCGCGGTATCNTGACCCTAA	287
RAT	1533	AGTACCCCATTGAACACGGCATTGTAACCAACTGGGACGATATGGAGAAGATTTGGCACC	1592
β-actin cDNA product	286	AGTATCNTATCGAGCATGGCATTGTCACCAACTGGGANGACATGGAAAAGATCTGGCACC	227
gp β-actin cDNA		TTGTTACCAACTGGGACGACATGGAGAAGATCTGGCACC	
RAT	1593	ACACTTTCTACAATGAGCTGCGTGTGGCCCCTGAGGAGCACCCTGTGCTGCTCACCGAGG	1652
β-actin cDNA product	226	ACACCTTTTACAACGAGCTGCGTGTGGCCCCTGAGGAGCACCCAGTGCTGCTGACAGAGG	167
gp β-actin cDNA		ACACCTTCTATAACGAGCTCC GTGTAGCTCCTGAGGAGCACCCCACCCTGCTCACCGAGG	
RAT	1653	CCCCTCTGAACCCTAAGGCCAACCGTGAAAAGATGACCCAGGTCA	1697
β-actin cDNA product	166	CCCCCTTGAACCCCAAGGCCAACAGAGAGAAGATGACTCAGATCA	122
gp β-actin cDNA		CCCCACTGAACCCCAAGGCCAACCGTGAGAAGATGAC	
RAT	2155	CAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTAGCCATCCAGGCTGTGTTG	2214
β-actin cDNA product	128	CAGATCATGTTTGAGACCTTCAACACCCCGGCCATGTACGTGGCTATCCAGGCTGTGNTN	69
RAT	2215	TCCCTGTATG	2224
β-actin cDNA product	68	TCCCTTTATG	59



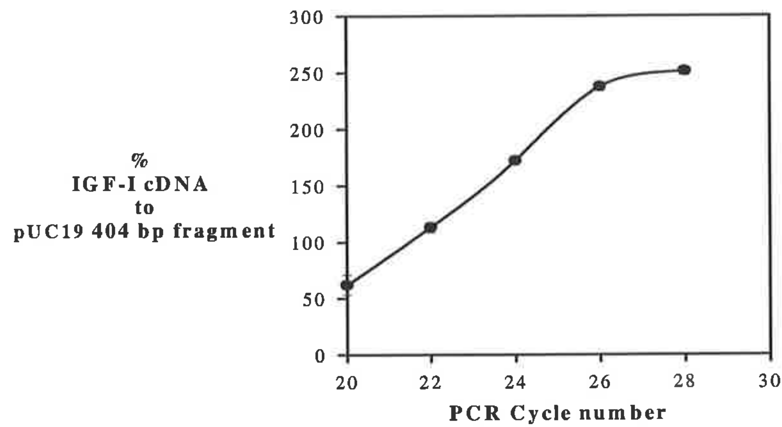
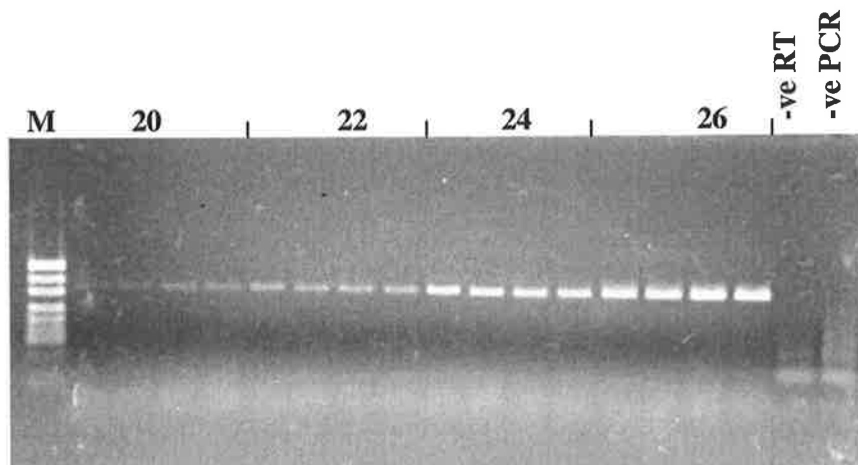


Figure 2.9 RT-PCR amplification profile of guinea pig liver IGF-I quantitated by agarose gel electrophoresis, ethidium bromide staining and transillumination with UV light.

Samples were resolved on a 2% agarose gel electrophoresed at 90V for 45 mins. The gel was stained with ethidium bromide and amplicons were visualised by UV light. M = 0.25 µg of pUC19 DNA/*Hpa II* standard. The numbers above the lanes indicate the number of PCR cycles. In the negative RT RNA was substituted with sterile water in the reaction mixture. In the negative PCR cDNA was replaced by sterile water in the reaction mixture. The density of amplified IGF-I cDNA fragment is expressed as a % of the pUC19 404 bp fragment.



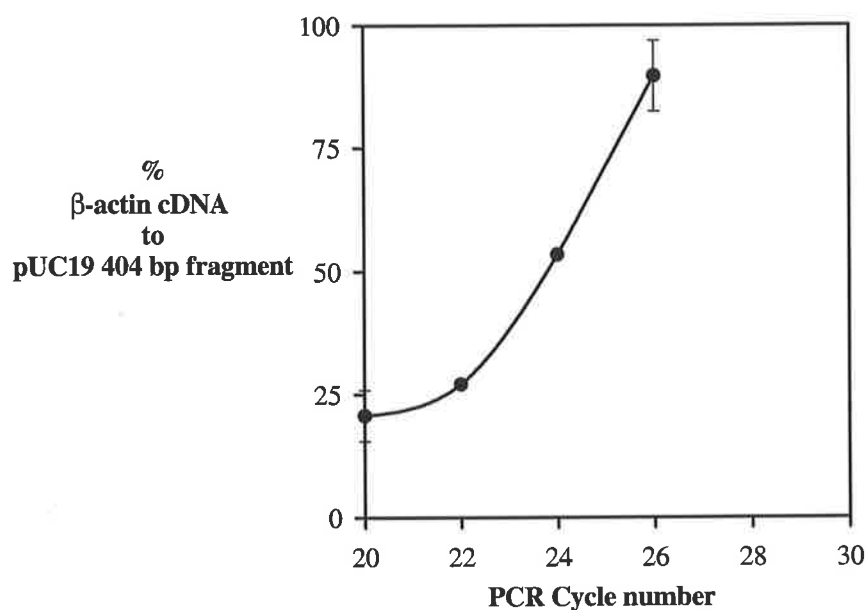


Figure 2.10 RT-PCR amplification profile of guinea pig liver β-actin quantitated by agarose gel electrophoresis, ethidium bromide staining and transillumination with UV light.

Samples were resolved on a 2% agarose gel electrophoresed at 90V for 45 mins. The gel was stained with ethidium bromide and amplicons were visualised by UV light. M = 0.25 μg of pUC19 DNA/*Hpa II* standard. The numbers above the lanes indicate the number of PCR cycles. In the negative RT RNA was substituted by sterile water in the RT reaction mixture. In the negative PCR cDNA was replaced by sterile water in the reaction mixture. The density of amplified β-actin cDNA fragment is expressed as a % of the pUC19 404 bp fragment.

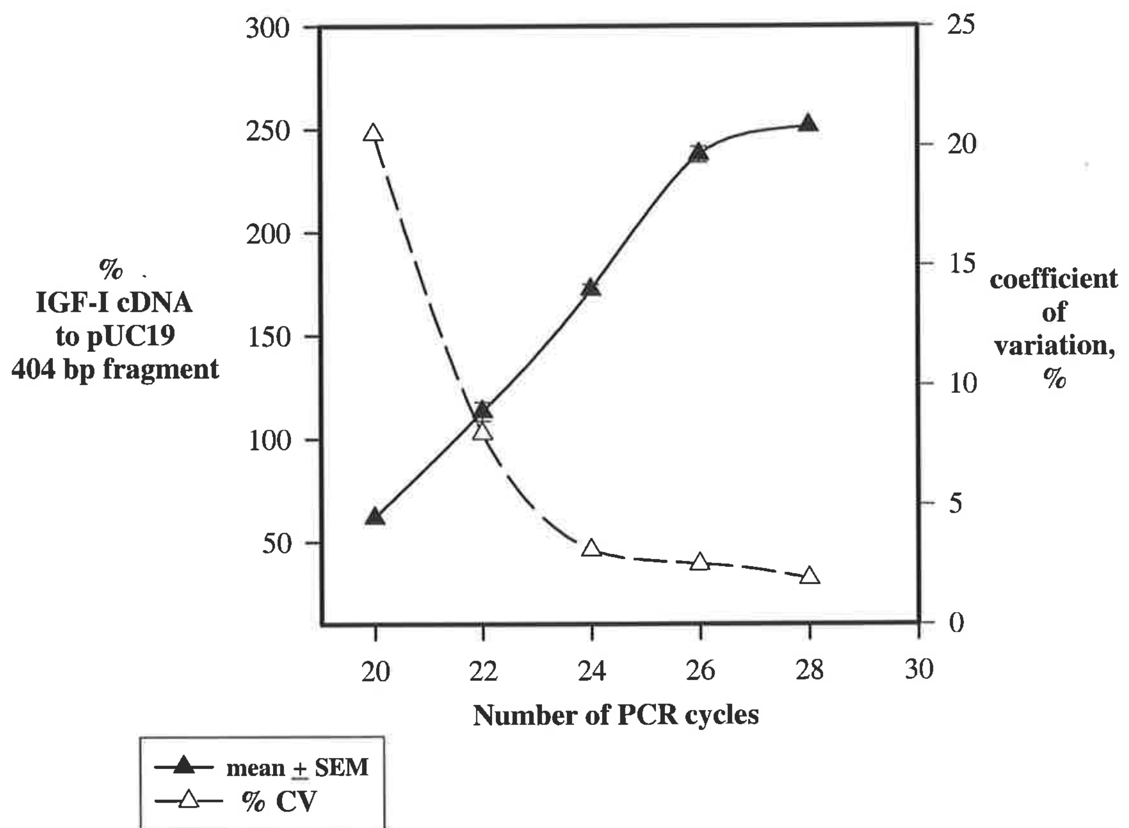


Figure 2.11 Variability of RT-PCR amplification of guinea pig liver IGF-I and the coefficient of variation associated with replicates run on the same gel and measured by densitometry.

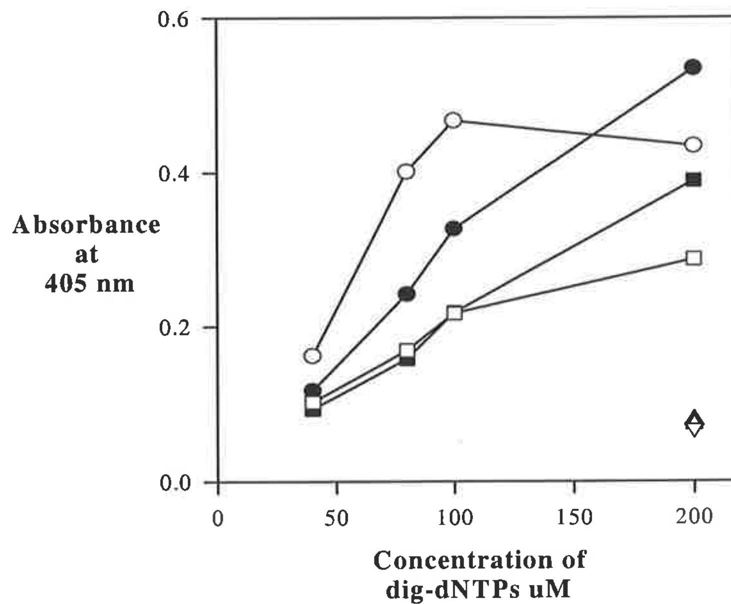
Amplification of IGF-I was assessed in duplicate for 20 and 28 cycles and in quadruplicate for 22, 24 and 26 cycles. The amount of amplified IGF-I fragment is expressed as a % of the pUC19 404 bp fragment and the mean ± sem is plotted.

variability was determined by using the density of the 404 bp pUC19/Hpa II DNA standard on three different gels and was found to be 16%.

2.3.5 Effect of concentration of cDNA template and digoxigenin-labelling reactants on Dig-ELISA estimation of IGF-I mRNA.

Digoxigenin labelling of IGF-I cDNA products was performed as in 2.2.5. The volume of reverse transcribed cDNA, concentration of Dig-dNTPs and volume of Dig-IGF-I cDNA PCR products added to the ELISA microtitre wells were evaluated to determine their effect on the Dig-ELISA estimation of IGF-I mRNA (Figure 2.12). The concentration of Dig-dNTPs that yielded maximal Dig-IGF-I labelled cDNA product from 2.5 µl of guinea pig liver cDNA preparation (equivalent to 0.125 µg of total RNA) was 100 µM when 10 µl of the Dig-IGF-I cDNA PCR product was measured in the dig ELISA. However, doubling the volume of guinea pig liver cDNA preparation to 5 µl (0.25 µg of total RNA) increased the incorporation of Dig-dNTPs at 200 µM which was the maximum concentration tested (Figure 2.12). The greater the incorporation of digoxigenin-dNTPs into IGF-I cDNA the greater the absorbance, irrespective of the amount of reverse transcribed cDNA initially amplified in the PCR reaction. A greater amount of bound hybrid (Dig-IGF-I cDNA hybridised to biotinylated gpIGF-I) was detected in 10 µl of amplified PCR products from 2.5 µl of liver cDNA than from 5 µl of liver cDNA at a concentration of 100 µM Dig-dNTPs (Figure 2.12). This may result from the presence of PCR inhibitors contaminating the cDNA preparation that may be affecting the polymerase enzyme reaction therefore reducing efficiency and dig incorporation rates.

Measurement in the Dig-ELISA was routinely performed by Dig-labelling 2.5 µl of guinea pig liver cDNA preparation (0.125 µg of RNA) using 100 µM Dig-dNTPs, followed by hybridisation of 10 µl of Dig-IGF-I PCR product to biotinylated gpIGF-I oligonucleotide, after



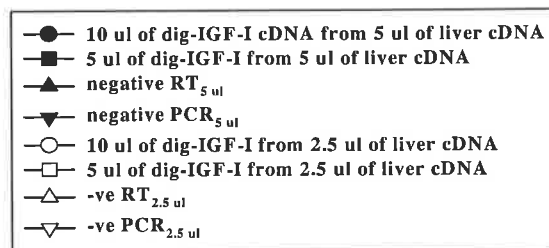


Figure 2.12 Effect of concentration of digoxigenin-dNTP and liver cDNA template on Dig-IGF-I cDNA measured in the Dig-ELISA

Immobilisation of biotinylated guinea pig IGF-I oligonucleotide hybridised to dig-IGF-I cDNA on a streptavidin-coated microtitre plate. Detection of bound hybrid was performed with peroxidase conjugated anti-digoxigenin antibody.
negative RT (sterile water substituted for RNA in reaction mixture)
negative PCR (sterile water substituted for cDNA in reaction mixture)
subscripts refer to the volume of sterile water

which 200 μl of the hybridised solution was added to the streptavidin-coated microtitre plate well.

2.3.6 Efficiency of amplification estimated by Dig ELISA

The average amplification efficiency estimated by Dig-ELISA was 64% for IGF-I after 22, 24 and 26 cycles inclusive and 53% for β-actin between cycles 18-20 inclusive (Figure 2.13). This was much greater than the amplification efficiencies for PCR assessed by densitometric analysis of ethidium bromide stained agarose gels (section 2.3.4). Detection of ethidium bromide stained PCR products for both amplicons was detectable from cycle 20.

The variability of Dig-labelling of cDNA in the PCR reaction using the same guinea pig liver cDNA was calculated to be between 5.4% and 20%. The within-assay variability was determined using a single Dig-labelled cDNA hybridised in triplicate in solution to the capture oligonucleotide and then each hybridised cDNA was measured in duplicate in the ELISA and the variability was found to be 3.2% ($n = 5$). The variability between three ELISA assays of the Dig-labelled IGF-I cDNA standard was found to be 14.6%. Comparison of the two methods for quantitation of mRNA has shown that there is greater variability at lower amplification cycles associated with gel analysis than that observed with the ELISA.

These results indicate that PCR Dig-ELISA can be used to quantify IGF-I mRNA and β-actin mRNA. The throughput of analyses per gel is limited to 16 and will be insufficient for the analysis of 31 test samples and standards. The coefficient of variation between gels was 43% indicating reproducibility was poorer. A major advantage of the ELISA system over gel analysis was that it allowed an increased throughput of 90 analyses, allowing comparisons between estimations of experiments with large numbers and reducing the associated variability.

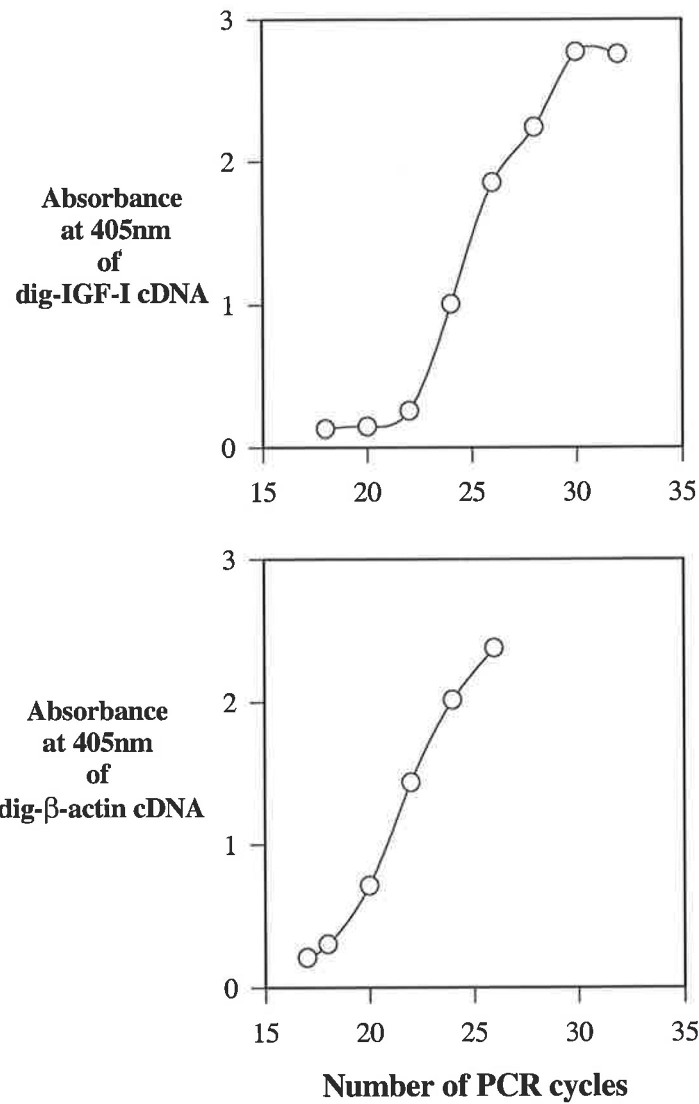


Figure 2.13 The efficiency of amplification of IGF-I and β-actin, measured by PCR Dig-ELISA

Amplification of dig-labelled PCR products are measured by the increase in absorbance at 405 nm in the ELISA.

2.3.7 Specificity

The specificity of the PCR ELISA was tested using a non-complementary capture oligonucleotide. A dig labelled PCR product produced using gp IGF-$I_{310/545}$ primers was hybridised to the biotinylated capture oligonucleotide for β-actin or that for IGF-I. A Dig-labelled PCR product produced using β-actin primers was treated similarly. All were then measured by Dig-ELISA (Table 2.4).

Table 2.4 Specificity of capture oligonucleotide for respective Dig labelled PCR amplicon.

	Absorbance at 405 nm in Dig-ELISA	
Dig labelled PCR product	**Capture oligonucleotide**	
	IGF-$I_{341/359}$	**β-$actin_{31/49}$**
gp IGF-$I_{310/545}$	1.4 ± 0.09	0.13 ± 0.002
r β-$actin_{1456/2291}$	0.13 ± 0.06	1.95 ± 0.008

2.3.8 Dig labelled IGF-I and β-actin calibration standards for Dig-ELISAs

The sizes of the Dig-labelled IGF-I and β-actin PCR products used as ELISA calibration standards were found to be 243.2 ± 2.5 bp and 383 ± 12.2 bp respectively by gel analysis. Their DNA concentrations were determined by electrophoresis and comparison with a known mass of pUC19/Hpa II DNA standard. IGF-I and β-actin PCR ELISA standards were serially diluted and the density of each measured after electrophoresis and ethidium bromide staining. The mass of IGF-I cDNA and β-actin cDNA as calculated from the plot of net intensity of pUC19/Hpa II DNA fragments versus the Log Mass (ng) of each standard Dig-amplicon. The DNA concentrations of 1:5, 1:10 and 1:20 quadruplicate dilutions of IGF-I Dig-ELISA standards were 16.3, 10.7, 12.7 mg/l respectively and the mean ± sem was 13.2 ± 2.0 mg/l. The mean DNA concentration of quadruplicate Dig-β-actin cDNA ELISA standard diluted 1:5, 1:7.5 and 1:10 were 1.5, 2.5, 3.9 mg/l respectively and the mean ± sem was 2.6 ± 0.7 mg/l.

2.4 DISCUSSION

RT-PCR is a sensitive, specific and quick nonisotopic method for the detection of mRNA. This method works very effectively with small amounts of RNA and total RNA preparations. Useful RNA can be successfully extracted from tissues if ribonuclease activity is inhibited or kept to a minimum. This can be achieved by the choice of reagents inhibiting RNases activity (guanidinium isothiocyanate) as well as using solutions and glassware that are RNase free and careful extraction to remove DNA, protein and lipid. Extraction of total RNA and the yield from various tissues varies widely and must be considered when embarking on a study involving the quantitation of mRNA by any method. The yield of RNA obtained from guinea pig liver in this study was reasonably reproducible. Extracted liver RNA had an $A_{260/280}$ ratio greater than 1.8 indicating the preparation had minimal protein and phenol contamination. Others have shown that total RNA extracted by **TRI**ZOL reagent gives consistent yields and high purity (Chadderton et al., 1997).

A single dsDNA product from liver, parametrial fat, interscapular fat and retroperitoneal fat of guinea pigs was obtained for both IGF-I and β-actin. The relative abundance of IGF-I mRNA estimated from ethidium stained agarose gels after RT-PCR of total RNA was qualitatively much greater in liver than in the three major fat sites measured. A previous study also showed that guinea pig liver abundantly expresses IGF-I mRNA compared with bone and cartilage (Gosiewska et al., 1994).

Use of guinea pig IGF-I specific primers permitted amplification of a product when sequenced was 100% identical to guinea pig IGF-I cDNA (Bell et al., 1990). Use of rat β-actin primersled to amplification of a product, which had 86% sequence homology with guinea pig β-actin (GenBank AF193571) and 86% homology with rat β-actin nucleotides 1473 to 1697

and 91% with nucleotides 2155 to 2224 (GenBank V01217). The discrepancy between the published sequence and the sequence of the amplified product could be explained by strain to strain variation in the sequence of guinea pig β-actin.

PCR reagent and cycling conditions for both IGF-I and β-actin were established and the efficiency of amplification estimated for two methods.

The successful performance of RT-PCR is dependent on many factors including reproducibility of reverse transcription. Often this step can introduce large variation. In this study the CV for reverse transcription ranged from 5.4% to 20%. For this reason an internal reference is required in the analysis.

Throughput and variance

Sample throughput limited the suitability of the method based on densitometric analysis of PCR products on ethidium bromide stained gels. A maximum of nine analyses in duplicate per gel can be performed. Approximately five gels would be required to analyse the same number than can be analysed by one Dig-ELISA. Two fold changes in levels of transcripts can be missed due to between gel variation. The Dig-ELISA allows 40 analyses per batch in duplicate minimising between-batch errors.

Efficiency

The efficiency of amplification determined by densitometric analysis of ethidium bromide stained bands after agarose gel electrophoresis for IGF-I between cycle 20 to 24 is 26% and between cycle 22 to 26 for β-actin was 34.5%. Using this method of analysis the efficiency of amplification was considerably low for each PCR reaction, thus contributing to quantitation error. Slight changes in amplification efficiency expand into greater changes in the amount of reaction product, leading to errors in accurate quantitation. If PCR is a 100% efficient then a

doubling of cDNA products occur per cycle however as efficiency for this study for the gel method is much less than 50%. In comparison the efficiency of amplification determined by ELISA for IGF-I between cycle 22 to 26 was 64% and for β-actin between cycle 18 to 20 was 53%. The efficiency of amplification was increased at least 2 fold for IGF-I and 1.5 times for β-actin when quantitation was performed by Dig-ELISA. Dig-ELISA estimation of Dig-cDNA products for either IGF-I or β-actin was far superior over the densitometric gel analysis. The small linear range and accuracy of measuring the intensity of the bands limit densitometric analysis of ethidium bromide stained gels which may account for the higher efficiencies observed for Dig-ELISA detection. As amplification increases the intensity of the bands increase but becomes indistinguishable in the exponential phase and the plateau phase. The ELISA can detect accurate changes in amplification as this detection method uses a specific capture oligonucleotide sequence and an enzyme detection system to quantify the PCR products.

Sensitivity

Sensitivity of both techniques is acceptable for this study. PCR products can be detected at cycle 20 for IGF-I by both detection systems, while β-actin was detected in the Dig-ELISA two cycles earlier. Gel analysis was performed on undiluted PCR product whereas Dig-β-actin PCR products were diluted for measurement in the ELISA. This suggests that the Dig-ELISA is a more sensitive method than the gel method. At cycle 20 IGF-I mRNA products were just visible on ethidium bromide stained agarose gel.

Specificity

Specificity of detection of amplified PCR products by the ELISA is potentially increased by having a third oligonucleotide "capture" probe, which is specific for the amplified product. Densitometric analysis of amplified PCR products measures the band at a predicted size and the identity of this product was verified by sequencing. Therefore the use of a third

oligonucleotide designed specifically for the gene of interest increases the specificity of the Dig-ELISA approach over that of the densitometric analysis of amplified PCR products.

Reproducibility

For densitometric analysis of ethidium bromide stained PCR products precision between replicate samples on the same gel was 3%, however, the between assay variability ranged from 16% to 43%. This variation was unsuitable for analyses aimed at detecting smaller differences between experimental treatment groups. Analysis of the variation of IGF-I cDNA product between cycle numbers 20 to 28 indicated that at lower cycle numbers the coefficient of variation was 20%, indicating the high variability in densitometric measurement. As cycle number increases and the cDNA product increases exponentially the coefficient of variation decreased to 1.9%. This suggests that the exponential phase of the reaction can be assessed accurately. In summary, densitometric analysis of ethidium stained agarose gel has good reproducibility if all samples are run on one gel but has large inter assay variation and therefore would not be ideal as an analytical tool for this study. In comparison the Dig-ELISA within-assay variation was 3.2% which was comparable with the gel method.

These results suggest that the Dig-ELISA can be used to quantify IGF-I mRNA and β-actin mRNA. Reverse transcription of up to 40 experimental samples should be performed in duplicate in a single batch, followed by a single PCR amplification batch and Dig-PCR products measured in a single Dig-ELISA assay. This will eliminate the variation associated with multiple RT and PCR batches. Quantitation of IGF-I cDNA and β-actin was therefore performed by Dig-ELISA. The main advantage of the Dig-ELISA over the gel densitometric analysis is the large throughput resulting in a lower level of within-assay variation.

In summary, guinea pig liver expresses more IGF-I mRNA than parametrial fat, interscapular fat and retroperitoneal fat, suggesting liver as a major source of IGF-I. A RT-PCR ELISA has

been developed and validated for the quantitative measurement of guinea pig IGF-I mRNA and β-actin mRNA.

CHAPTER 3

THE EFFECT OF PREGNANCY AND NUTRITION ON HEPATIC EXPRESSION OF IGF-I: ROLE OF THE IGF ENDOCRINE SYSTEM IN PREGNANCY

3.1 INTRODUCTION

In humans and in other mammals IGF-I concentrations in maternal blood are related to fetal body weight and size (Caufriez et al., 1994;McIntyre et al., 2000). In mothers carrying IUGR fetuses maternal plasma IGF-I was lower than normal and correlated with fetal weight (Lassarre et al., 1991). High plasma IGF-I in pregnant mice abolished the negative relationship seen between fetal weight and litter size (Gluckman et al., 1992), suggesting IGF-I may have a role in enhancing nutrient availability or placental capacity to deliver nutrients. IGF-I has been suggested to act as a regulator of maternal metabolism during pregnancy. IGF-I promotes glucose and amino acid uptake and inhibits protein degradation in muscle (Oddy and Owens, 1996). Infusion of IGF-I into pregnant ewes stimulates placental lactate production, which is taken up by the fetus (Liu et al., 1994). IGF-I also has anabolic effects on maternal tissues in pregnant rats (Gargosky et al., 1991). Therefore, circulating IGF-I may play an important role in regulating maternal metabolism and partitioning substrates to supply fetal and/or placental growth.

Circulating IGF-I levels increase progressively with advancing pregnancy in humans and guinea pigs (Gargosky et al., 1990a;Sohlstrom et al., 1998). In rodents IGF-I increases during the first half and then declines in the second half of pregnancy (Gargosky et al., 1990b). Pregnancy also increases IGFBP-3 protease activity in blood in rats and humans, which potentially alters the bioavailability of IGF-I and its clearance from the circulation. In humans and rats proteolytic modification of IGFBP-3 by pregnancy specific proteases lowers the binding affinity for IGFs affecting bioavailability for target tissues (Baxter, 1994;Blat et al., 1994;Lassarre and Binoux, 1994). In guinea pig plasma, pregnancy specific proteases for IGFBP-3 have not yet been identified (Sohlstrom et al., 1998). However tissue specific

proteases may be present similar to those identified in human placental trophoblast cells and decidua, affecting the availability of IGFs to selected tissues (Irwin et al., 2000;Rajah et al., 1995).

Liver is known to be the major source of endocrine IGF-I in nonpregnant rats. However it is uncertain whether the increase in plasma IGF-I seen early in pregnancy in rats is due to increased hepatic production or due to increased clearance of IGF-I (Davenport et al., 1990;Monaco and Donovan, 1997). Hepatic abundance of IGF-I mRNA increased in sows treated with GH in the second quarter of pregnancy and this increase was associated with increased maternal plasma IGF-I (Sterle et al., 1998).

The effect of pregnancy on endocrine IGF-I is similar in humans and guinea pigs where pregnancy progressively increases concentrations of IGF-I and IGF-II is also present in relatively large amounts. Preliminary studies (section 2.3.2) indicated that liver is the major site of IGF-I synthesis in guinea pigs. This study therefore measured IGF-I mRNA in liver using the RT-PCR dig-ELISA developed in chapter 2. The aim was to determine the effects of pregnancy and nutrition on IGF-I expression. The effects of pregnancy and nutrition on relationships between hepatic abundance of IGF-I mRNA, plasma IGFs and IGF binding proteins and pregnancy phenotype were also investigated.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Design

Nulliparous female guinea pigs (IMVS coloured strain) aged 3 to 4 months weighing 472 ± 56 g (n = 31) were from the Gilles Plains Animal Resource Centre SA. Animals were given unrestricted access to a guinea pig/rabbit ration supplemented with vitamin E (165 mg/kg) which was prepared by Milling Industries Stockfeeds, Murray Bridge. Water containing vitamin C (400 mg/l) was freely available. Animals were housed under a 12 hours light and dark cycle with temperature maintained at 25°C.

The animals used in this study were a subset of those described in Sohlstrom *et al* 1998, where plasma IGFs and body composition for the whole cohort were reported. Guinea pigs were allocated to four treatments (Figure 3.1). They were either fed *ad libitum* (n=8) or 30% less than *ad libitum* (per g body weight) for 28 days prior to mating and for the first 34 days of pregnancy, then 10% less than *ad libitum* (per g body weight) for the next 26 days (n=8). Term is 69 days. The attenuation of feed restriction over the last 26 d of pregnancy minimises fetal loss. Food intake and body weight were measured three times per week. The maternal weight gain and net maternal weight gain (maternal weight minus the weight of the uterus containing fetal and placental tissue) was taken from day 1 of pregnancy to day 60 of pregnancy. Nonpregnant animals were weighed for the same time period as for the pregnant animals. Nonpregnant guinea pigs were similarly fed (*ad libitum*, n=5; feed restricted, n=10) for 88 days. Pregnant animals were humanely killed on day 60 of pregnancy and nonpregnant guinea pigs after 88 days of the experimental diets.

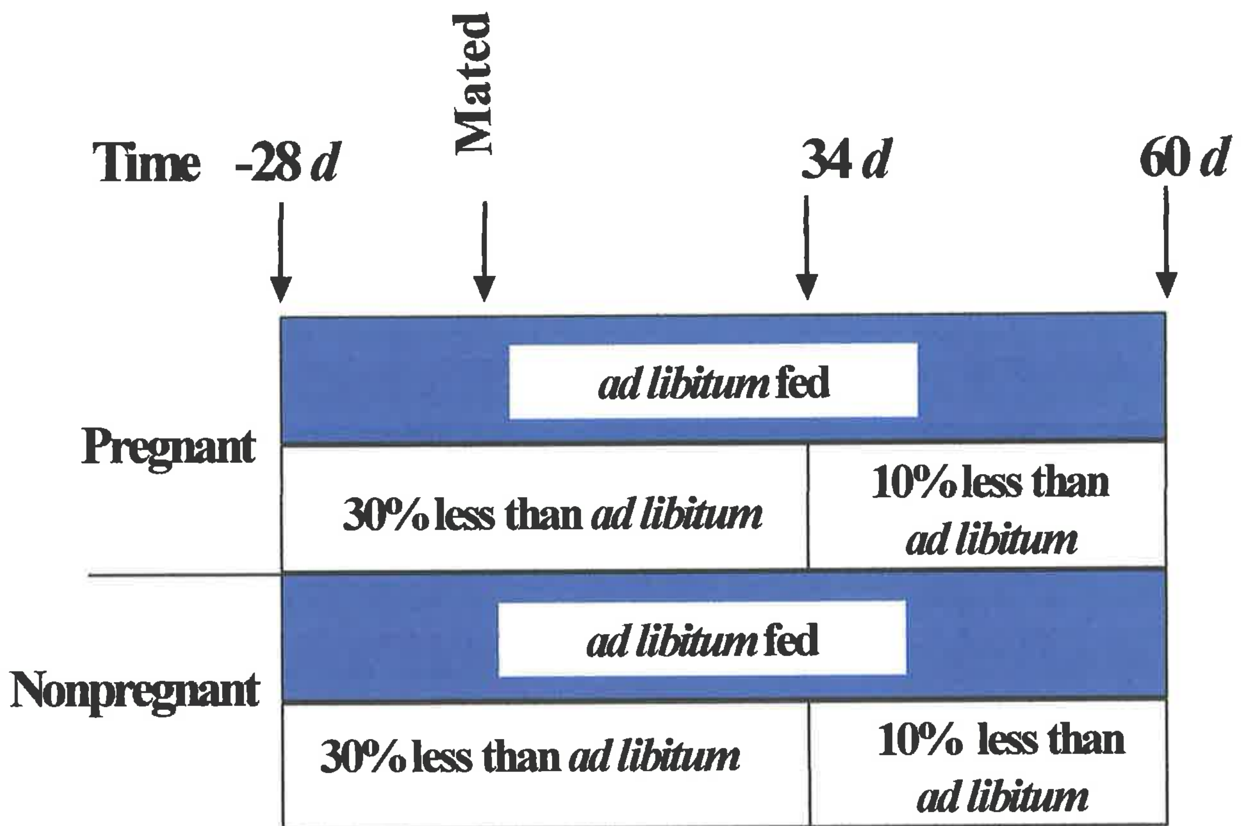


Figure 3.1. Schematic representation of nutritional treatments of pregnant and nonpregnant guinea pigs.

Animals were fed either *ad libitum* or 30% less than *ad libitum* per kg body weight for 28 days prior to mating. Nutritionally restricted animals were maintained on the 30% less than *ad libitum* for 62 days after which the diet was altered to 10% less than *ad libitum*. Animals were euthanased at day 60 of pregnancy (term is 69 days).

3.2.2 Collection of blood and tissues

The Adelaide University Animal Ethics Committee approved this study design. Animals were killed by an intraperitoneal overdose of pentobarbital sodium. Blood (10 ml) was collected immediately by cardiac puncture into a heparinised syringe and stored on ice until centrifugation at 4°C for 10 mins at 2500 rpm (J-6B Beckman Instruments USA). Plasma was stored at -20°C. The following tissues were immediately removed weighed and snap frozen: adrenals, kidneys, spleen, pancreas, liver, gastrointestinal tract, retroperitoneal fat, interscapular fat, parametrial fat, heart, lungs, thymus, thyroids as well as the biceps brachii, soleus, and gastronemius muscles. All tissues were stored at –80°C. All fetal and placental weights were also determined.

3.2.3 IGF-I Radioimmunoassay

Acid column chromatography of plasma samples

Plasma IGF-I and IGF-II were measured by radioimmunoassay after removal of IGFBPs by size exclusion high performance liquid chromatography (HPLC) at pH 2.5 (Owens et al., 1990). Plasma samples (90 μl) were diluted in 360 μl water, acidified with 150 μl of 4 times concentrated HPLC mobile phase, mixed and incubated at room temperature for 30 mins. The acidified solution was defatted by mixing with an equal volume of freon (1,1,2-trichloro-1, 2, 2-trifluoroethane). The organic and aqueous phases were separated by centrifugation at 10 000 rpm for 10 mins at 4°C. A portion of the upper aqueous phase (400 μl) was filtered through a 0.45 μm cellulose acetate micro-spin filter membrane (cat no. 2485, Alltech Associates Inc.) by centrifugation at 10 000 rpm for 3 mins at 4°C. Two hundred μl of filtrate, equivalent to 30 μl of plasma, was injected (Shimadzu SIL-9A, Japan) onto a Protein-Pak 125 HPLC size exclusion column (Waters Millipore, Lane Cove, NSW, Australia) equilibrated at 1 ml/min with 200 mM acetic acid, 50 mM trimethylamine, 0.05% Tween-20 at pH 2.5

(mobile phase). The recovery from the Protein-Pak 125 column was estimated by injecting ~20 000 cpm of radiolabelled IGF-I and counting the eluted radioactivity. Recovery was 95 ± 0.6 % (n= 8). Four fractions of eluate were routinely collected for each acidified plasma specimen. Fraction 1 was collected between 6.25 to 8.25 mins (2.0 ml), fraction 2 from 8.25 to 8.75 mins (0.5 ml), fraction 3 from 8.75 to 10.75 mins (2.0 ml), and fraction 4 from 10.75 to 11.25 mins (0.5 ml) after injection. Quality control was maintained by the inclusion of pooled guinea pig plasma, which had been stored in 500 μl aliquots at –20°C, and was extracted and chromatographed as above at least once per HPLC run. An average of 15 test specimens were injected at intervals of 30 mins in each HPLC batch run. A large pool of chromatographed pig plasma, previously eluted in fraction 3 from many HPLC runs, was also used as a quality control specimen in every RIA. Routinely, all four fractions from the pooled guinea pig plasma (chromatographed quality control) and randomly selected test specimens, fraction 3 for the remaining test specimens ($15 < n < 20$) and the pig plasma pooled eluate were included in every RIA of IGF-I and IGF-II.

Measurement of IGF-I by radioimmunoassay

Recombinant human IGF-I (Gropep Pty. Ltd, Adelaide) was used to prepare the standard and radiolabelled ligand for RIA. IGF-I was iodinated using chloramine-T and $Na^{125}I$ (Gargosky et al., 1990a;Owens et al., 1990). Specific radioactivity of [^{125}I]-iodo-IGF-I was 80 Ci/ g. Rabbit anti-human IGF-I antibody (MAC Ab 89/1) was used at a final dilution of 1:60 000. IGF-II has less than 1% of the potency of IGF-I in this assay (Francis et al., 1989a;Francis et al., 1989b). Guinea pig IGF-I and IGF-II are identical to the human polypeptides. All measurements were performed in triplicate.

One hundred μl of the chromatography fraction was added to a polystyrene tube, followed by two hundred μl of RIA buffer (30 mM Na_2HPO_4; 0.02% (w/v) protamine sulphate; 10 mM disodium EDTA; 0.05% (v/v) Tween-20; 0.02% (w/v) NaN_3; pH 7.5) and 60 μl of 0.4 M Tris

base to bring the pH to 7.4. A stock solution of 10 ng/ml IGF-I standard in RIA buffer stored at –20°C was serially diluted in IGF RIA buffer to prepare a standard of < 5 to 500 pg/tube which was added in 200 µl. Mobile phase (100 µl) and 60 µl of 0.4 M Tris base was added to each standard tube as neutralized eluate blank. Anti-human IGF-I (50 µl) and 50 µl of radioiodinated h-IGF-I (20 000 cpm) were added per tube. Tubes containing 50 µl radiolabelled IGF-I only, provided a measure of total radioactivity added and tubes with RIA buffer and radiolabelled IGF-I only provided a blank (no IGF-I antiserum). The tubes were mixed and incubated for 22 hrs at 4°C. Ten µl of a 1:20 dilution in RIA buffer of rabbit IgG (cat no. P0448, DAKO, Australia Pty Ltd) and 50 µl of a 1:20 dilution in RIA buffer of sheep anti-rabbit IgG (cat no. RC, Silenus, Victoria, Australia) were added. The tubes were mixed and incubated for 30 mins at 4°C. One ml of ice cold polyethylene glycol 6000 (6% w/v) in 150 mM of aqueous NaCl was added and the tubes were centrifuged at 4000 rpm for 25 mins at 4°C (J-6B Beckman, Instruments USA). The supernatant was aspirated and radioactivity in the pellet was measured in a gamma scintillation spectrometer (1261 MULTIGAMMA, LKB Pharmacia, and Wallac Oy). The RiaCalc II data management program (Pharmacia, Wallac Oy) calculated IGF-I concentrations and the minimal detectable concentration, which was 6.5 pg/tube on average. This is equivalent to 4.9 ng of IGF-I per ml of plasma. The coefficient of variation for the guinea pig plasma pool was 2 %. The intra and inter assay coefficients of variation, assessed by repeated measures of the pig pool in the RIA, were 5.5% and 7.8% respectively.

Measurement of IGF-II by radioimmunoassay

Recombinant human IGF-II receptor grade (Gropep Pty. Ltd.) was used for preparation of the standard and radiolabelled ligand. IGF-II was iodinated using chloramine-T and $Na^{125}I$ (Owens et al., 1990). Specific activity of the radio-ligand was 70 Ci/ g. A mouse anti-rat

IGF-II monoclonal antibody (Dr K Nishikawa, Kanazawa, Medical University, Ishikawa, Japan) was used at a final dilution of 1: 500 in RIA buffer. IGF-I has 2.5% of the activity of IGF-II in this RIA (Carr et al 1995/6). All measurements were performed in triplicate. Fifty μl of the appropriate HPLC fraction was added to a polystyrene tube followed by RIA buffer (200 μl), and 30 μl of 0.4 M Tris base to bring the pH to 7.4. A stock solution of 10 ng/ml IGF-II standard in RIA buffer stored at –20°C was serially diluted in IGF RIA buffer for use in generating the standard of < 5 to 500 pg/tube which was added to RIA tubes in 200 μl. Mobile phase (50 μl) and 30 μl of 0.4 M Tris base were added per standard tube as neutralized eluate blank. Anti-rat IGF-II (50 μl) and 50 μl of radioiodinated h-IGF-II (20 000 cpm) were added to each tube. Tubes with radiolabelled IGF-II provided a measure of total counts and tubes with RIA buffer and radiolabelled IGF-II (no IGF-II antiserum) provided a measure of the blank. The tubes were mixed and incubated for 22 hrs at 4°C. Ten μl of a 1:20 dilution in RIA buffer of mouse serum (IMVS, Gilles Plains, South Australia) and 50 μl of a 1:20 dilution in RIA buffer of sheep anti-mouse IgG (cat DS, Silenus, Victoria, Australia) were added. The tubes were mixed and incubated for 30 mins at 4°C. One ml of ice cold polyethylene glycol 6000 (6% w/v) in 150 mM of aqueous NaCl was added and the tubes were centrifuged at 4000 rpm for 25 mins at 4°C (J-6B Beckman, Instruments USA). The supernatant was aspirated and the radioactivity in the pellet was measured in a gamma counter. The minimal detectable concentration (see IGF-I RIA above) was 19 pg/tube. The intra and inter assay coefficient of variation were 7.8% and 10.1% respectively.

Measurement of IGFBPs by western ligand blot analysis.

IGF-binding proteins in plasma were measured by western ligand blotting (Hossenlopp et al., 1986). Ten μl of guinea pig plasma was diluted with 90 μl of water and 100 μl of 4 x sodium dodecyl sulfate (SDS) gel loading buffer (0.25 M Tris base, 8% SDS w/v, 20% glycerol v/v

and 0.04% bromophenol blue w/v). Samples were heated at 65^0C for 15 mins before loading 20 μl (equivalent to 1 μl guinea pig plasma) onto a non-reducing discontinuous sodium dodecyl sulfate-polyacrylamide gel with a 4% stacking gel and a 10% separating gel prepared as follows.

The separating gel was prepared by mixing 15 ml of lower gel buffer (1.5 M Tris base, 4 % SDS, pH 8.8), 15 ml of a 40% solution of acrylamide (w/v) (17-1303-01, Pharmacia, Biotech), 8 ml of 2% methylene-bis acrylamide (w/v) solution (17-1306-01, Parmacia, Biotech), 22 ml of water, 105 μl of tetramethylethylenediamine (TEMED) and 105 μl of 10% solution of ammonium persulphate (APS). The separating gel solution was poured between the sandwiched glass plates and overlaid with water. After polymerization the water was aspirated. The stacking gel, which was prepared by mixing 5 ml of upper gel buffer (0.5 M Tris base, 4% SDS, pH 6.8), 2.2ml of 40% (w/v) acrylamide, 1.2 ml of 2% (w/v) methylene-bis acrylamide, 11.6 ml of water, 100 μl of 10% APS (w/v) and 20 μl of TEMED, was poured over the separating gel and a 10 well comb was inserted. After polymerisation of the stacking gel the comb was removed and the wells were filled with running buffer (0.025 M Tris base, 0.192 M glycine and 1% SDS).

Included on each gel were Rainbow™ [^{14}C] methylated protein molecular weight markers 14.3 to 220 kDa (cat CFA 756, Amersham Life Science, Buckinghamshire, England) Rainbow™ [^{14}C] was diluted 1 in 2 with 4 x SDS loading buffer and aliquots of 10 μl are frozen at -20°C for routine use. A pooled guinea pig plasma sample, made up of equal volumes of plasma from all treatment groups and divided into 50 μl aliquots, was also stored at –20°C and included on each gel. The molecular weight of IGFBPs was estimated by comparison with the electrophoretic mobility of the Rainbow™ standards. Electrophoresis was performed for 2 hours at 20 mA followed by 18 hrs at 8 mA in running buffer.

The proteins were transferred onto nitrocellulose membranes (0.45 μm) by electroelution (Schleicher and Schuell, Dassel, Germany) at 250 mA for 3.5 hours in tris/glycine buffer (0.2 M Tris base, 0.15 M glycine and 20% ethanol v/v). Nitrocellulose membranes were prepared by shaking for 30 mins in 250 ml of washing buffer (2 M NaCl, 0.5 M Tris base) containing 1% (v/v) Triton X-100 before use.

Following electroelution membranes were incubated in 250 ml of washing buffer containing 1g/l bovine serum albumin (BSA) for 90 mins at 21°C, shaking at 160 rpm. The membranes were then soaked for 10 mins in washing buffer containing 0.1%(v/v) Tween. Radiolabelled ^{125}I-IGF-II (10,000,000 cpm) was added to 250 ml of washing buffer containing 1 g/l of BSA and the membranes were incubated for 2.5 hours, shaking at 160 rpm at 23°C. Membranes were then washed for 30 mins x 3 with fresh wash buffer containing 0.1% (v/v) Tween. Membranes were airdried overnight at 23°C and were exposed at -80°C with intensifying screens to duplicate Fujifilm X-ray film (RX, Fuji Photo Film Co, Ltd, Tokyo). One was exposed for one week to measure IGFBP-3 and the other was exposed for two weeks to measure the other IGFBPs. The X-ray films were scanned (Image Quant, version 3.22, Computing Densitometer, model 300A, Molecular Dynamics, Sunnyvale, CA) and the density of each electrophoretic band was measured and expressed as a % of the corresponding band in the pooled guinea pig plasma reference that was included in all gels and blots.

3.2.4 Isolation of RNA

Total RNA was extracted from livers of 35 guinea pigs as described in section 2.2.2. These extractions were performed over 4 days. RNA was extracted from a designated reference liver on each day and livers from each of the treatment groups were extracted each day. Extraction

of RNA from 9 experimental livers and the reference liver was performed on days 1 and 4 and from 8 experimental livers and reference liver on days 2 and 3. Tissue pieces used for RNA extraction ranged from 81 to 322 mg wet weight. The integrity of RNA for each liver extract was assessed as described in section 2.2.2. Two μl of loading buffer was added to 20 μl of RNA (2 μg) in molecular grade water and 11 μl was loaded per well of an agarose gel which was equivalent to 1 μg of RNA per liver. Ten μl of a RNA marker, 0.363 kb to 9.5 kb (cat no. IB76200, Scientific Imaging Systems, Eastman Kodak Co., New Haven, CT), was loaded into one well per gel. The density of the 28S and 18S ribosomal RNA was quantified by comparison with the density of the 3.911 kb fragment of the RNA size markers after staining with ethidium bromide and UV transillumination.

Each RNA extract was divided into two aliquots, one stored at -80°C in ethanol and the other in aqueous 5 M NaCl as described in section 2.2.2. The second aliquot was further diluted with molecular grade water to a final concentration of 0.1 μg/μl and stored at -80°C. The diluted RNA solutions were used for reverse transcription to produce cDNA.

3.2.5 Reverse Transcription of RNA from experimental guinea pig livers

Reverse transcription was performed as described in 2.2.3 in duplicate batches containing each test and control RNA. All 0.1 μg/μl RNA solutions were thawed on ice simultaneously and 20 μl of the RNA solution (equivalent to 2 μg of RNA) was transferred to 0.5 ml flat cap PCR tubes (cat no. 13320-00 Astral Scientific, Gymea, NSW). After the addition of 2 μl of random hexamers (100 μg/ml) (section 2.2.3) in sterile water the tubes were incubated at 65°C for 10 mins then placed on ice for 5 mins. A master mix sufficient for 42 reactions (168 μl of 100 mM dithiothreitol, 336 μl of 5 x cDNA synthesis buffer (250 mM Tris-HCl, 200 mM KCl, 25 mM $MgCl_2$, 2.5%Tween^{R}20, pH 8.3), 168 μl of 10 mM dNTPs and 84 μl of

Expand^{TM}RT enzyme) was prepared. After cooling, 18 µl of the master mix was added per tube. A negative control was also included which contained water instead of RNA. The reaction solutions were then heated to 30°C for 10 mins, 42°C for 45 mins followed by 95°C for 2 mins. The reaction solutions were placed on ice and diluted to 160 µl by adding 120 µl of molecular grade water. The cDNA preparations were then stored at -20°C. These reverse transcribed products are designated $cDNA_1$. The above reverse transcription was repeated for all RNA solutions on a separate day, resulting in duplicate cDNA preparations (designated $cDNA_2$) for each liver RNA extract. The solutions of RNA were frozen and thawed once for the duplicate RT ($cDNA_2$).

3.2.6 Dig-labelling of IGF-I and β-actin cDNA from guinea pig liver by PCR

All $cDNA_1$ solutions were subjected to digoxigenin labelling PCR. Ten µl of cDNA preparation from each liver (equivalent to 0.125 µg of RNA) was added to flat cap PCR tubes. Molecular grade water (10 µl) replaced cDNA as negative controls. Forty µl of a solution sufficient for 44 reactions containing 110 µl of 2mM Digoxigenin-dNTPs, 176 µl of 25 mM $MgCl_2$, 220 µl of 10x Taq reaction buffer, 220 µl of gpIGF-I_{310} oligonucleotide primer (5 µmol), 220 µl of gpIGF-I_{545} oligonucleotide primer (5 µmol), 220 µl Taq Polymerase 0.2 U/µl and 594 µl of molecular grade water was added to each PCR tube. PCR reactants were heated to 94°C for 3 mins followed by 24 repeated cycles of denaturation at 94°C for 15 secs, annealing at 58°C for 15 secs, extension at 72°C for 45 secs. A final extension at 72°C for 5 mins was performed after 24 cycles and the products were frozen at -20°C. IGF-I cDNA products from $cDNA_1$ are referred to as dig-IGF-I_1. Digoxigenin labelling of IGF-I cDNA was performed on duplicate $cDNA_2$ reverse transcription product, referred to as dig-IGF-I_2.

Amplification and dig-labelling of β-actin cDNA was also performed. Molecular grade water (10 μl) was substituted for cDNA as a negative control and 10 μl of the reverse transcribed water solution (RT negative control) was also included in the PCR batch. Forty μl of a solution sufficient for 44 reactions containing 110 μl of 2 mM Digoxigenin-dNTPs, 176 μl of 25 mM $MgCl_2$, 220 μl of 10x Taq reaction buffer, 220 μl of rβ-$actin_{1456}$ oligonucleotide primer (5 μmol), 220 μl of rβ-$actin_{2291}$ oligonucleotide primer (5 μmol), 220 μl Taq Polymerase 0.2 U/μl and 594 μl of molecular grade water was added to each PCR tube. PCR reactants were heated to 94°C for 3 mins followed by 21 repetitive cycles of denaturation at 94°C for 15 secs, annealing at 58°C for 15 secs, extension at 72°C for 45 secs. A final extension at 72°C for 5 mins was performed after 21 PCR cycles and the products were stored at -20°C. Digoxigenin labelled β-actin cDNA products are denoted dig-β-$actin_1$ or dig-β-$actin_2$ depending on whether they originated from reverse transcription products $cDNA_1$ and $cDNA_2$ respectively. The relative abundance of IGF-I mRNA in guinea pig livers was expressed as the ratio of dig-IGF-I to dig-β-actin cDNA products derived from RNA extracted from the same liver. Figure 3.2 summarises steps 3.2.4 to 3.2.7.

3.2.7 Measurement of guinea pig hepatic IGF-I and β-actin Dig-labelled cDNA by enzyme linked immunosorbent assay

Frozen dig-labelled IGF-I $cDNA_1$ and negative controls were thawed on ice and diluted 1:25 with ELISA solution containing biotinylated IGF-I oligonucleotide (section 2.2.9). The mixtures were firstly heated at 94°C for 10 mins, then hybridisation of the biotinylated IGF-I capture oligonucleotide (section 2.2.9) to the dig-labelled IGF-I cDNA was permitted at 60°C for 10 mins followed by cooling to 23°C for 10 mins. Once cooled, 200 μl aliquots of each solution were added in duplicate to wells of a streptavidin-coated microtitre plate. Serial dilutions of quantitated standards for dig-labelled IGF-I (section 2.2.9.) were included. The

standard dig-labelled IGF-I (0.25, 0.5, 1, 2, 4 μl of 13.2 ng/μl) was diluted in ELISA solution and allowed to hybridise to the biotinylated IGF-I oligonucleotide as above. Section 2.3.8 describes the quantitation of the dig-IGF-I ELISA standards. The diluted dig-labelled standards (200 μl) were added to the plate in duplicate. On each microtitre plate duplicate wells containing 200 μl of ELISA solution only (blank) were also added. After standards, unknowns and controls are added to the microtitre plate wells, the plate was covered and incubated at 37°C for 3 hrs shaking at 160 rpm. The plate was washed 3 times with wash buffer (section 2.2.9). The anti-digoxigenin-antibody polyperoxidase conjugate was diluted 1:3750 in conjugate buffer (section 2.2.9), 200 μl added per well and incubated at 37°C for 30 mins shaking at 160 rpm. The micotitre plate was washed 3 times with wash buffer. Two hundred μl of ABTS solution (section 2.2.9) was added per well and incubated in the dark at 37°C for 30 mins shaking at 160 rpm. Dig-IGF-I_2 cDNA products were measured as described above. Dig-β-$actin_1$ and dig-β-$actin_2$ cDNA products were measured as above except a biotinylated gp β-actin oligonucleotide (section 2.2.9) was used at 50 ng/ml. The spectrophotometric absorbance was measured at 405 nm. The relative abundance of IGF-I mRNA was expressed as a ratio of dig-IGF-I / dig-β-actin cDNA products derived from the same hepatic cDNA. Figure 3.2 summarises steps 3.2.4 to 3.2.7.

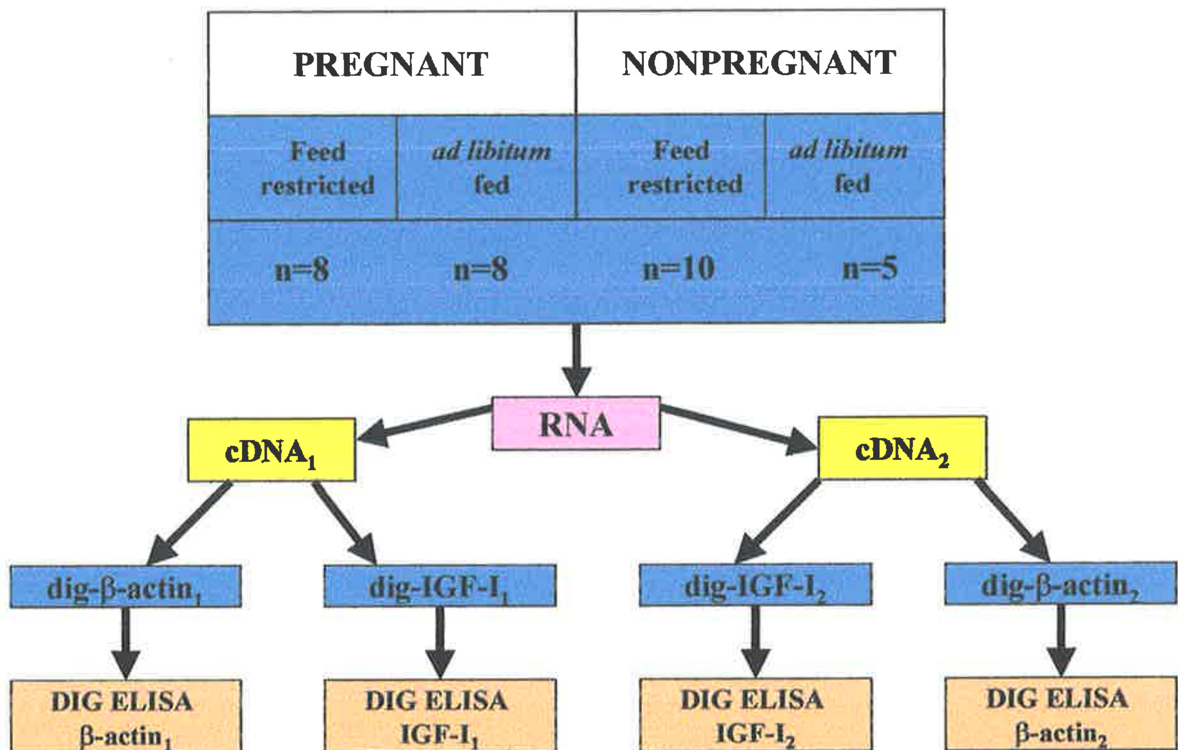


Figure 3.2. Flow chart representing the processing of all liver samples from RNA extraction to RTPCR and DIG ELISA for the quantitation of IGF-I mRNA and β-actin mRNA.

Total RNA was extracted from 31 liver samples. RNA (2 ug) extracted from each liver was reverse transcribed (RT) twice in independent reverse transcription batches. Each RT product $cDNA_1$ or $cDNA_2$ was subjected to PCR amplification with digoxigenin-nucleotide triphosphates to produce dig-β-$actin_1$, dig-β-$actin_2$, dig-IGF-I_1 and dig-IGF-I_2. Dig-β-actin cDNA products were measured in two DIG β-actin ELISAs. Dig-IGF-I cDNA products were quantified in two DIG IGF-I ELISAs. The average of dig-IGF-I_1 and dig-IGF-I_2 and dig-β-$actin_1$ and dig-β-$actin_2$ were used to determine the amounts of dig-labelled PCR products derived from each liver RNA extract.

3.2.8 Statistics

Data are presented as mean ± sem. The effects of pregnancy, nutrition and their interaction were assessed by two way ANOVA. Comparison between experimental groups was performed using Student-Newman-Keuls Method. Associations were evaluated by polynomial regression (SigmaStat, Jandel, San Raael, CA, USA). The effect of pregnancy and nutrition on plasma IGF-I was assessed by one way ANOVA. The effect of nutrition on fetal and placental weights was assessed by one way ANOVA. Pearson product moment correlation was employed to measure the parametric strength of associations between two variables. Co-variate analysis was performed using forward stepwise linear regression to identify the independent variables. Multiple linear regression was then performed to best describe the data when two or more variables were identified. Simple linear regression was used when one independent variable was identified. The software package used for all analyses was SigmaStat (Jandel Scientific, San Raael, CA, USA).

3.3 RESULTS

3.3.1 Maternal and fetal phenotype

Adult

Pregnancy did not increase food intake (as a fraction of body weight) in *ad libitum* fed guinea pigs compared to nonpregnant animals as previously reported (Sohlstrom et al., 1998) (dta not shown). Pregnancy increased body weight ($p<0.001$) in *ad libitum* fed and feed restricted guinea pigs (Table 3.1). Feed restriction reduced body weight ($p<0.001$) in pregnant and nonpregnant guinea pigs (Table 3.1). Maternal net body weight (total body weight minus that of the uterus, fetuses and placenta), net weight gain (total weight gain minus the weight of the uterus, fetuses and placenta for pregnant animals) and carcass weight (eviscerated carcass) and carcass fractional weight (expressed as a % of body weight) were lower in feed restricted pregnant and nonpregnant animals ($p<0.001$). Pregnancy increased weight gain (weight gained from day 1 to day 60 of pregnancy) in *ad libitum* and feed restricted guinea pigs ($p<0.001$). Undernutrition decreased weight gain ($p<0.001$) in nonpregnant and pregnant animals.

Pregnancy increased absolute uterine weight (consisting of uterus, fetuses and placentae) and fractional uterine weight expressed both as a % of body weight ($p<0.001$) and of carcass weight ($p<0.001$) (Table 3.2). Undernutrition significantly decreased absolute uterine weight in pregnant animals and fractional uterine weight expressed either as a % of body weight ($p<0.005$) or as a % of carcass weight ($p<0.001$) (Table 3.2). Liver weight ($p<0.001$) was lower in feed restricted pregnant and nonpregnant animals. Undernutrition decreased the weight of spleen, retroperitoneal fat and dorsal fat in pregnant and nonpregnant animals but

(g)	Non Pregnant *Ad libitum* n = 5	Non Pregnant Restricted n = 10	Pregnant *Ad libitum* n = 8	Pregnant Restricted n = 8	p
Body weight	744.6 ± 27.7	505.6 ± 19.6	957.4 ± 21.9	656.8 ± 21.9	**F** p <0.001, **P** p<0.001
Net body weight	743.5 ± 24.1	504.8 ± 17.1	712.6 ± 19.1	547.3 ± 19.1	**F** p <0.001
Weight gain	185.6 ± 16.5	19.7 ± 11.7	383.5 ± 13	136.4 ± 13	**F** p <0.001, **P** p<0.001 **F** x **P** p<0.01
Net weight gain	184.5 ± 17.3	18.9 ± 12.3	138.7 ± 13.7	26.9 ± 13.7	**F** p <0.001
Carcass	372.6 ± 13.5	244.2 ± 9.5	367.6 ± 10.7	257.2 ± 10.7	**F** p <0.001
Carcass %	50.1 ± 1.2	48.4 ± 0.8	51.6 ± 0.9	47.1 ± 0.9	**F** p = 0.005

Table 3.1 Effect of pregnancy and nutrition on maternal phenotype. Expressed as mean ± sem. Effects of treatment were assessed by two-way ANOVA with pregnancy status and nutritional regimen as between factors. All pairwise multiple comparison procedures were performed by Student-Newman-Keuls Method. Net body weight (weight minus the uterus, fetal and placental tissues), carcass weight (consisting of skin, bone and unweighed muscle) and carcass % (expressed as a % of body weight). **P** = pregnant, **F** = feed

Tissue weight (g)	Non Pregnant *Ad libitum* n = 5	Non Pregnant Restricted n = 10	Pregnant *Ad libitum* n = 8	Pregnant Restricted n = 8	p
Uterus, g	1.09 ± 13.98	0.82 ± 9.8	244.7 ± 11	109.4 ± 11.05	**F** p<0.001, **P** p<0.001 **F** x **P** p<0.001
Uterus as % of body weight	0.15 ± 2.4	0.16 ± 1.7	34.5 ± 1.9	20.2 ± 1.9	**F** p<0.005, **P** p<0.001 **F** x **P** p<0.005
Uterus as % of carcass weight	0.29 ± 5.3	0.33 ± 3.7	67.2 ± 4.2	43.4 ± 4.2	**F** p<0.001, **P** p<0.001 **F** x **P** p<0.001
Liver, g	35.1 ± 1.4	21.7 ± 0.9	31.2 ± 1.1	22.4 ± 1.1	**F** p<0.001
Liver as % of body weight	4.7 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	4.1 ± 0.1	**F** p<0.01, **P** p<0.05
Liver as % of carcass weight	9.4 ± 0.4	8.9 ± 0.3	8.5 ± 0.3	8.7 ± 0.3	NS

Table 3.2 Effect of pregnancy and feed availability on maternal tissue weights. Expressed as mean ± sem. Effects of treatment were assessed by two-way ANOVA with pregnancy status and nutritional regimen as between factors. All pairwise multiple comparison procedures were performed by the Student-Newman-Keuls Method. Uterine weight in pregnant animals is the combined weights of the uterus, fetuses and placenta.
P = pregnancy, **F** = feed

Tissue weight (g)	Non Pregnant *Ad libitum* n = 5	Non Pregnant Restricted n = 10	Pregnant *Ad libitum* n = 8	Pregnant Restricted n = 8	p
Retroperitoneal fat	7.41 ± 0.4	0.49 ± 0.3	6.32 ± 0.32	0.6 ± 0.32	**F** $p<0.0001$
Dorsal fat*	11.8 ± 0.75	2.1 ± 0.53	9.93 ± 0.59	2.7 ± 0.59	**F** x $p<0.0001$ **F** x **P** $p<0.05$
Total gastrointestinal tract	34.6 ± 2.2	32.2 ± 1.6	28.4 ± 1.8	36.1 ± 1.8	**F** x **P** $p< 0.02$
Spleen	1.47 ± 0.07	0.75 ± 0.05	1.42 ± 0.06	0.78 ± 0.06	**F** $p<0.0001$
Thymus	0.48 ± 0.03	0.18 ± 0.02	0.29 ± 0.02	0.1 ± 0.02	**P** $p<0.0001$, **F** $p<0.0001$ **P x F** $p<0.05$
Soleus muscle	0.36 ± 0.01	0.29 ± 0.01	0.39 ± 0.01	0.32 ± 0.01	**F** $p<0.005$, **P** $p<0.0001$

Table 3.3 Effect of pregnancy and feed availability on maternal tissue weights. Effects of treatment were assessed by two-way ANOVA with pregnancy status and nutritional regimen as between factors. All pairwise multiple comparison procedures were performed by the Student-Newman-Keuls Method. Results expressed as mean ± sem.
P = pregnancy, **F** = feed

this effect was smaller in dorsal fat of pregnant animals (Table 3.3). Pregnancy decreased total gastrointestinal tract weight in *ad libitum* fed animals only. Feed restriction increased total gastrointestinal tract weight in pregnant guinea pigs but not in nonpregnant animals. Pregnancy decreased thymus weight in *ad libitum* and feed restricted animals. Undernutrition ($p<0.0001$) decreased thymus weight in both pregnant and nonpregnant animals but this effect was smaller in pregnant guinea pigs. Pregnancy ($p<0.0001$) increased soleus muscle weight in feed restricted guinea pigs. Undernutrition ($p<0.005$) reduced soleus muscle weight in nonpregnant animals.

Fetus and placenta

Total fetal weight (total weight of fetuses per dam), total placental weight (total weight of placentae per dam), average fetal weight (mean weight of fetuses per dam), average placental weight (mean weight of placentae per dam) and average fetal to placental weight ratio (mean weight of fetuses per dam divided by the mean weight of placentea per dam) were positively correlated with pregnant uterine weight in *ad libitum* and feed restricted guinea pigs combined (Figure 3.3). Undernutrition decreased average fetal weight ($p<0.001$), average placental weight ($p<0.001$) and average fetal to placental weight ratio ($p<0.005$) (Table 3.3). Total fetal weight was twice as high in *ad libitum* fed mothers, although litter size was unaffected in my study. Total placental weight was reduced in feed restricted animals (Table 3.4). *Ad libitum* feeding increased average fetal weight by 67%, average placental weight by 36%, total placental weight by 92% and average fetal to placental weight ratio by 21%. Total fetal weight expressed as a fraction of total maternal body weight or carcass weight and total placental weight as a fraction of total body weight were higher in the *ad libitum* fed mothers (Table 3.4). However, total placental weight expressed as a fraction of carcass weight was unaffected by nutrition.

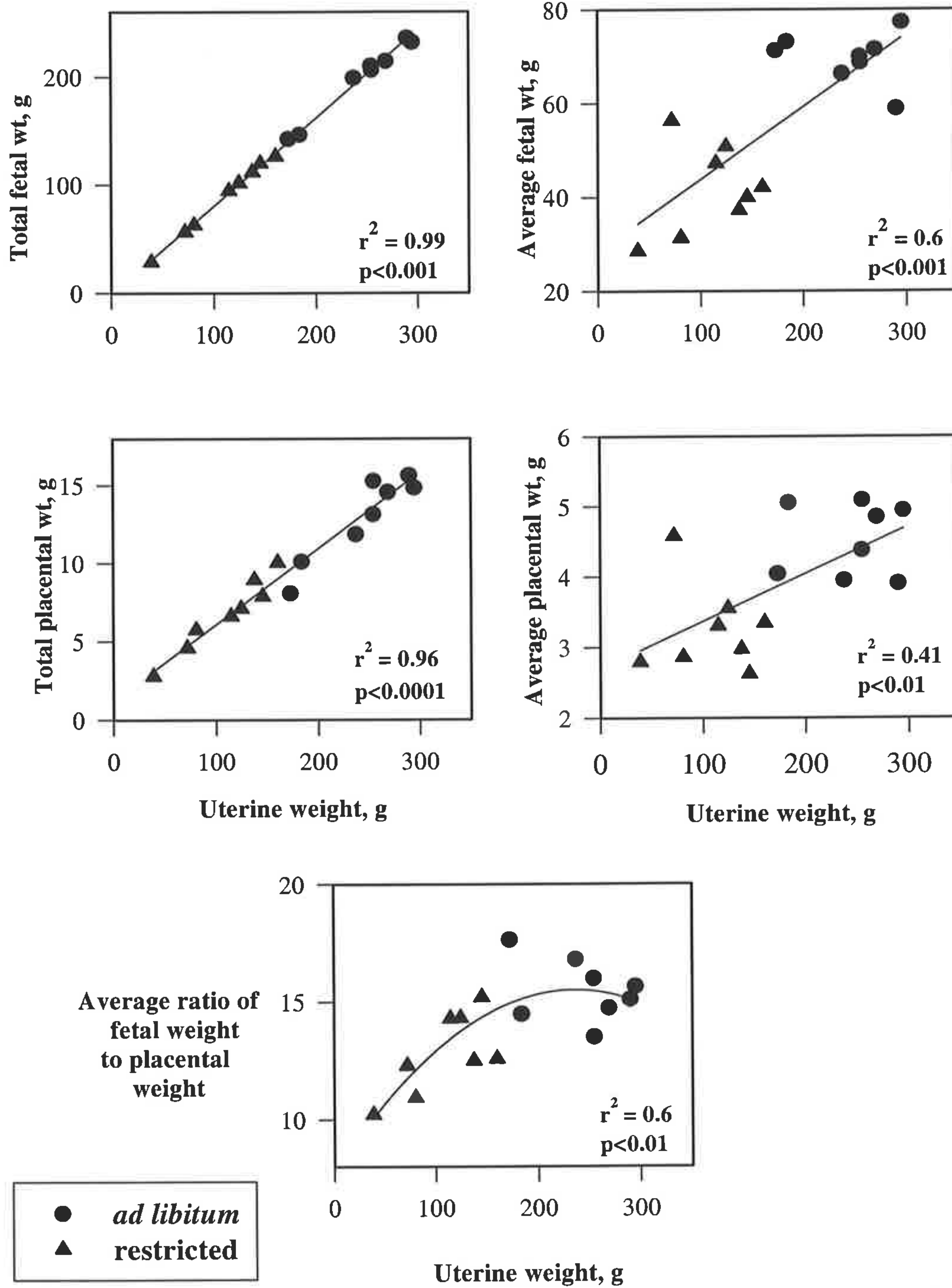


Figure 3.3 Relationship between pregnant uterine weight, fetal and placental weights.
The r^2 value is given for either a linear or polynomial relationship.

Table 3.4 Effect of nutrition on fetal and placental weights at day 60 of pregnancy. Values are mean ± sem. Data were analysed by one-way ANOVA with nutrition as the between factor.

	***Ad libitum* fed**	**Restricted**	**p value**
Total uterine weight	244.8 ± 16	109.4 ± 14.7	p<0.001
Fetuses per dam	2.9 ± 0.3	2.1 ± 0.3	
Total fetal weight, g	198.3 ± 12.5	87.7 ± 12.3	p<0.001
Total fetal weight as % body weight	20.7 ± 1.1	13.2 ± 1.7	p<0.005
Total fetal weight as % carcass	54.5 ± 4.1	34.8 ± 5.3	p<0.02
Total placental weight, g	12.9 ± 0.9	6.7 ± 0.8	p<0.001
Total placental weight as % body weight	1.35 ± 0.08	1.01 ± 0.11	p<0.05
Total placental weight as % carcass	3.55 ± 1.3	2.7 ± 0.4	
Average fetal weight, g	69.7 ± 1.9	41.7 ± 3.4	p<0.001
Average placental weight, g	4.5 ± 0.18	3.3 ± 0.2	p<0.01
Average fetal /placental weight ratio	15.5 ± 0.5	12.8 ± 0.6	p<0.005

3.3.2 Effect of pregnancy and nutrition on circulating IGF-I and IGF-II

Concentrations of IGF-I in adult plasma ranged from 32 to 923 ng/ml. Plasma IGF-I was increased by pregnancy ($p<0.001$) in *ad libitum* fed animals. Undernutrition decreased plasma IGF-I in pregnant guinea pigs only ($p<0.001$) (Figure 3.4). Plasma IGF-II concentrations were unaffected by pregnancy, but undernutrition reduced IGF-II by ≈ 50% in both pregnant and non-pregnant animals (Figure 3.5).

3.3.3 Effect of pregnancy and nutrition on circulating IGFBPs

Pregnancy increased plasma IGFBP-1 in feed restricted but not *ad libitum* fed animals. Undernutrition decreased plasma IGFBP-1 in nonpregnant guinea pigs but increased IGFBP-1 in pregnant animals. Pregnancy increased plasma IGFBP-2 in nutritionally restricted animals only. Feed restriction increased IGFBP-2 in pregnant animals only. Plasma IGFBP-3 was unaffected by pregnancy, but was reduced by more than 50% by undernutrition in pregnant and nonpregnant animals (Figure 3.6). Pregnancy increased IGFBP-4 in *ad libitum* fed and feed restricted animals (Table 3.5). Nutrition had no effect on IGFBP-4. IGFBP-3 proteolytic activity was not observed after mixing plasma from nonpregnant guinea pigs with plasma from pregnant guinea pigs.

3.3.4 Effect of pregnancy and nutrition on the ratio of IGF to IGFBPs

Pregnancy increased the ratio of IGF-I to IGFBP-1 in *ad libitum* fed animals but decreased this in feed restricted guinea pigs. Undernutrition decreased the ratio of IGF-I to IGFBP-1 in pregnant animals only. Pregnancy increased the ratio of IGF-I to IGFBP-2 in *ad libitum* fed

animals and was decreased in feed restricted guinea pigs (Table 3.6). Feed restriction reduced the ratio of IGF-I to IGFBP-2 in pregnant, but not in nonpregnant animals.

Pregnancy increased the ratio of IGF-I to IGFBP-3 in *ad libitum* fed animals. Nutritional restriction decreased the ratio of IGF-I to IGFBP-3 in pregnant animals only. Pregnancy had no effect on the ratio of plasma IGF-I to plasma IGFBP-4. Undernutrition reduced the circulating ratio of IGF-I to IGFBP-4 in pregnant and nonpregnant animals.

Pregnancy decreased the ratio of plasma IGF-II to IGFBP-1 in feed restricted animals only. Undernutrition reduced the circulating ratio of IGF-II to IGFBP-1 in pregnant but not in nonpregnant guinea pigs. The ratio of IGF-II to IGFBP-2 was unaffected by pregnancy. Food restriction decreased the ratio of IGF-II to IGFBP-2 in pregnant and nonpregnant animals. The ratio of plasma IGF-II to IGFBP-3 was unaffected by pregnancy or nutrition. Pregnancy decreased the ratio of IGF-II to IGFBP-4 in *ad libitum* and feed restricted animals. Undernutrition reduced the ratio of plasma IGF-II to IGFBP-4 in pregnant and nonpregnant guinea pigs.

	Non Pregnant		Pregnant		
	Ad libitum n = 5	Restricted n = 10	*Ad libitum* n = 8	Restricted n = 8	p
IGF-I, ng/ml	268 ± 27	213 ± 48	730 ± 35	240 ± 57	# **F** p <0.001, **P** p<0.001
IGF-II, ng/ml	1566 ± 94	790 ± 71	1614 ± 133	701 ± 127	**F** p <0.001
IGFBP-1, % of reference	230 ± 36	112 ± 18	239 ± 30	316 ± 33	**P** p<0.001 **F** x **P** p<0.005
IGFBP-2, % of reference	90 ± 12	118 ± 17	108 ± 10.6	224 ± 19	**F** p <0.001, **P** p<0.001 **F** x **P** p<0.02
IGFBP-3, % of reference	217 ± 14	90 ± 12	223 ± 16.7	101 ± 11	**F** p <0.001
IGFBP-4, % of reference	145 ± 43	121 ± 14	215 ± 23.6	306 ± 63	**P** p<0.005

Table 3.5 Effects of pregnancy and feed availability on IGF endocrine variables. Results expressed as mean ± sem. Effect of treatment was assessed by two-way ANOVA with pregnancy status and nutritional regimen as between factors. # indicates effects of treatments were assessed by one-way ANOVA. P = pregnancy, F = feed.

Ratio of the concentrations in plasma of	Non Pregnant *Ad libitum* n = 5	Non Pregnant Restricted n = 10	Pregnant *Ad libitum* n = 8	Pregnant Restricted n = 8	p
[IGF-I]/[IGFBP-1]	1.3 ± 0.4	2.1 ± 0.3	3.4 ± 0.3	0.89 ± 0.3	**F** p <0.02 **F** x **P** p<0.001
[IGF-I]/[IGFBP-2]	3.3 ± 0.9	2.5 ± 0.7	7.3 ± 0.8	1.12 ± 0.8	**F** p <0.001 **F** x **P** p<0.005
[IGF-I]/[IGFBP-3]	1.3 ± 0.5	2.3 ± 0.4	3.4 ± 0.4	2.7 ± 0.4	**P** p<0.02 **F** x **P** p<0.05
[IGF-I]/[IGFBP-4]	2.6 ± 0.6	1.7 ± 0.4	3.6 ± 0.5	1.4 ± 0.5	**F** p <0.005
[IGF-II]/[IGFBP-1]	7.5 ± 1.5	8.6 ± 1.0	7.7 ± 1.2	2.7 ± 1.2	**P** p <0.05 **F** x **P** p<0.05
[IGF-II]/[IGFBP-2]	18.9 ± 2.4	8.1 ± 1.7	16.5 ± 1.9	3.1 ± 1.9	**F** p<0.001
[IGF-II]/[IGFBP-3]	7.4 ± 1.7	9.8 ± 1.2	7.5 ± 1.3	8.2 ± 1.3	
[IGF-II]/[IGFBP-4]	14.9 ± 2.0	6.7 ± 1.5	8.0 ± 1.6	3.8 ± 1.6	**P** p<0.01, **F** p<0.002

Table 3.6 Effects of pregnancy and feed availability on the ratio of the concentration of IGFs to IGFBPs. Treatment was assessed by two-way ANOVA with pregnancy status and nutritional regimen as between factors.
The dependent variable is a derived measurement of the ratio of plasma IGFs to plasma IGFBPs.

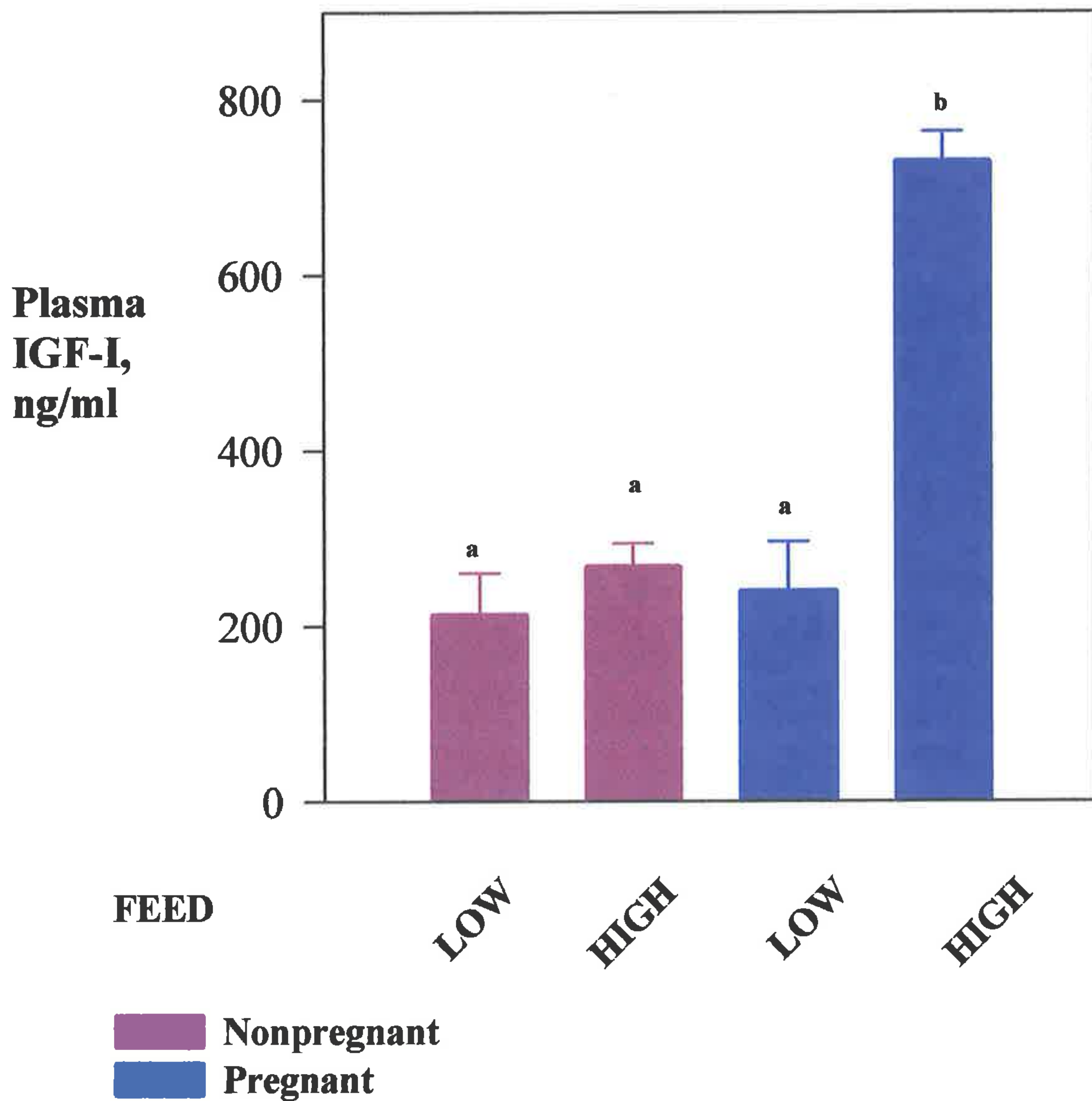


Figure 3.4 Effect of pregnancy and nutrition on plasma IGF-I in guinea pigs. Animals were provided *ad libitum* (HIGH) or restricted (LOW) access to feed. Different letters depict the one-way ANOVA results $p<0.001$.

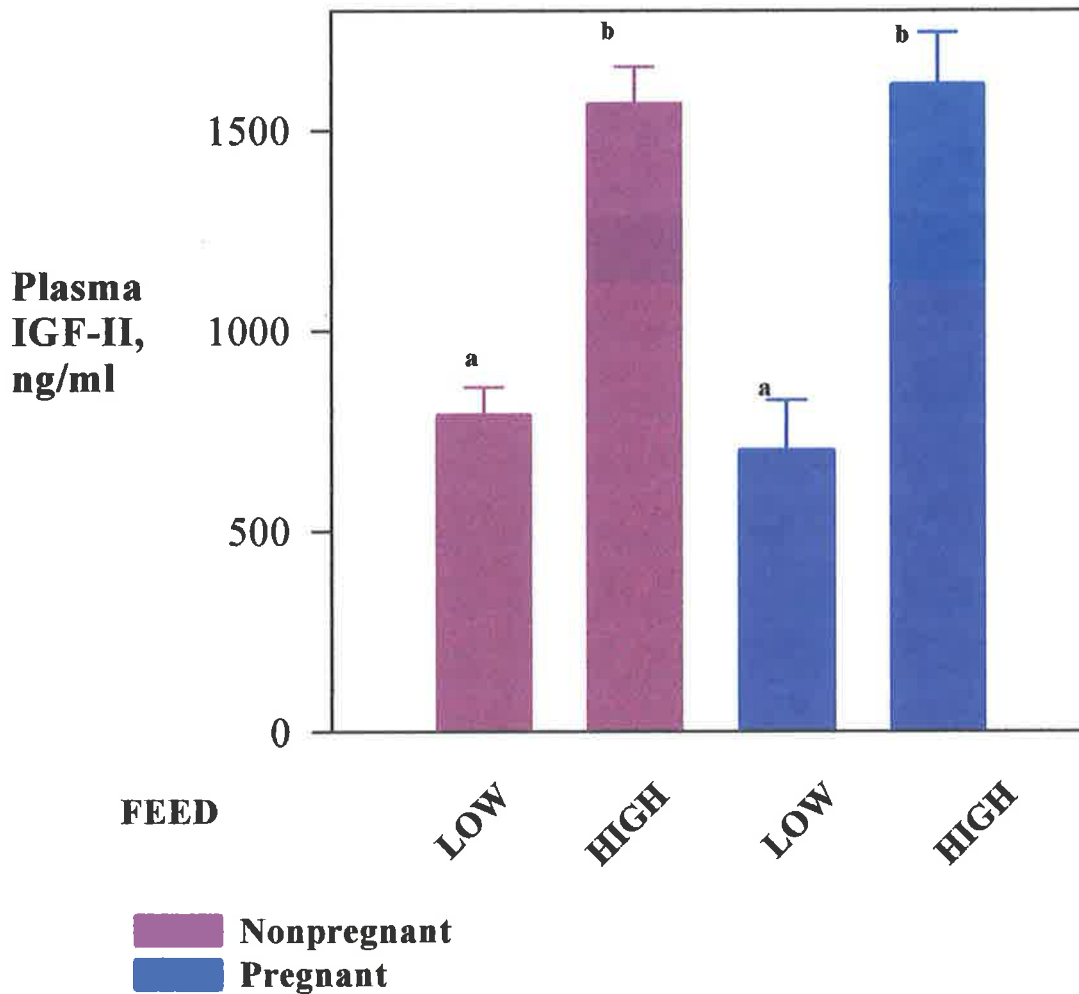


Figure 3.5 Effect of pregnancy and nutrition on plasma IGF-II in guinea pigs. Animals were provided *ad libitum* (HIGH) or restricted (LOW) access to feed. Different letters depict two-way ANOVA results $p<0.001$.

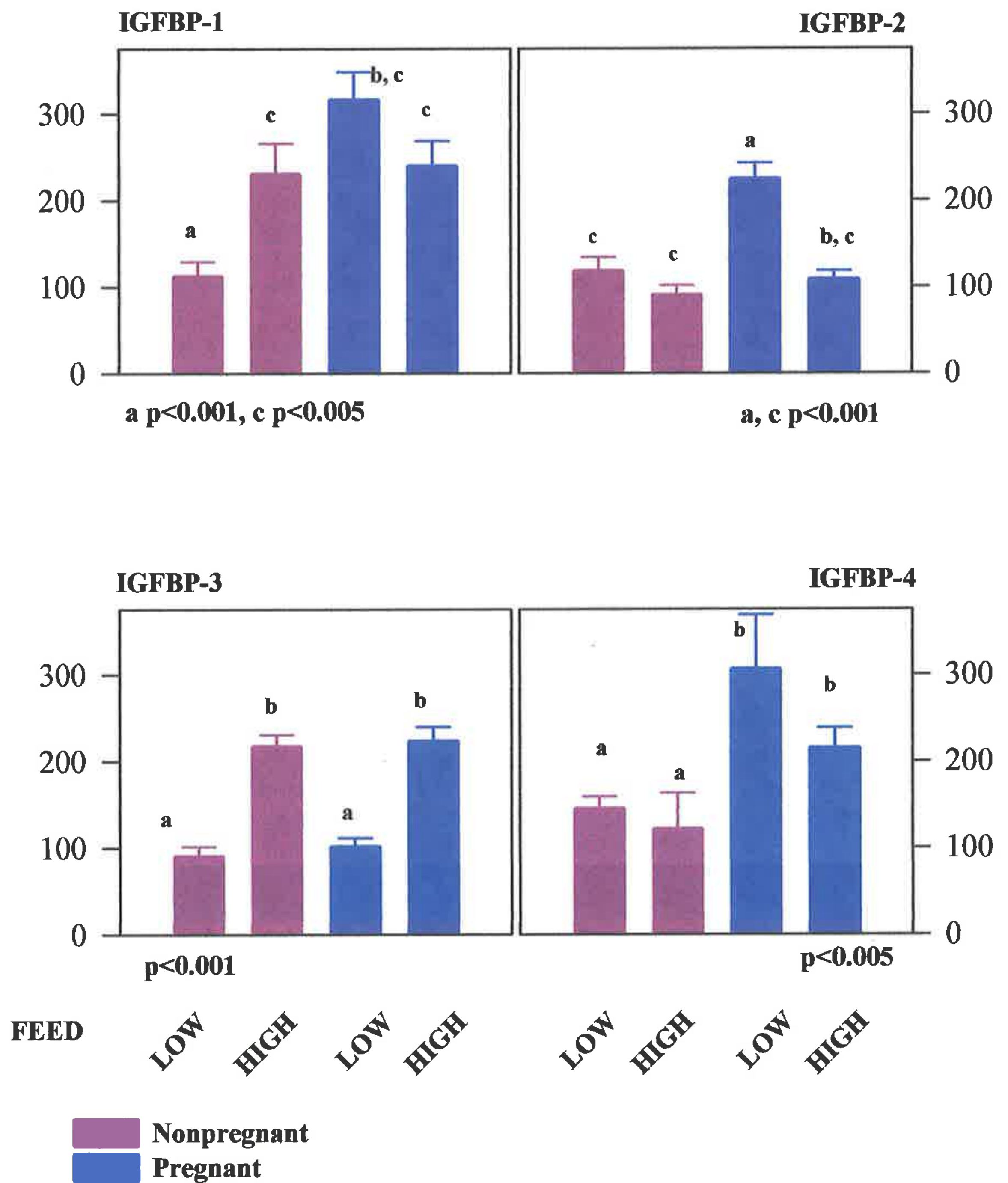


Figure 3.6 Effect of pregnancy and nutrition on plasma IGFBPs in guinea pigs. Animals were provided *ad libitum* (HIGH) or restricted (LOW) access to feed. Different letters depict two-way ANOVA results, p values are on each graph. IGFBP concentrations are expressed as % of a reference described in section (3.2.3).

3.3.5 Effect of pregnancy and nutrition on total RNA content of liver

Guinea pig liver RNA extracted from all experimental tissues had intact 18S and 28S ribosomal RNA subunits with minimal degradation (Figure 3.7). None of the RNA extracted from guinea pig livers was excluded from the study.

The total amount of RNA per liver was calculated by:

$$\text{Total liver RNA (mg)} = \text{RNA yield (mg/g)} \times \text{liver weight (g)}$$

There were no differences in the yield of RNA mg/g of tissue from any of the treatment groups (Figure 3.8). The amount of total liver RNA in *ad libitum* fed pregnant (49.2 ± 4 mg) and nonpregnant guinea pigs (45.6 ± 5.06 mg) was significantly greater ($p<0.0001$) than in food restricted pregnant (30.8 ± 4 mg) and nonpregnant (26.4 ± 3.58 mg) guinea pigs.

Ad libitum nutrition increased total liver size and total RNA content but not RNA content per g of tissue when compared to feed restriction. The mean yield of RNA extracted from the control liver (Figure 3.8) in the four RNA batch extractions was (1.08 ± 0.18 mg/g), a coefficient of variation of 35%.

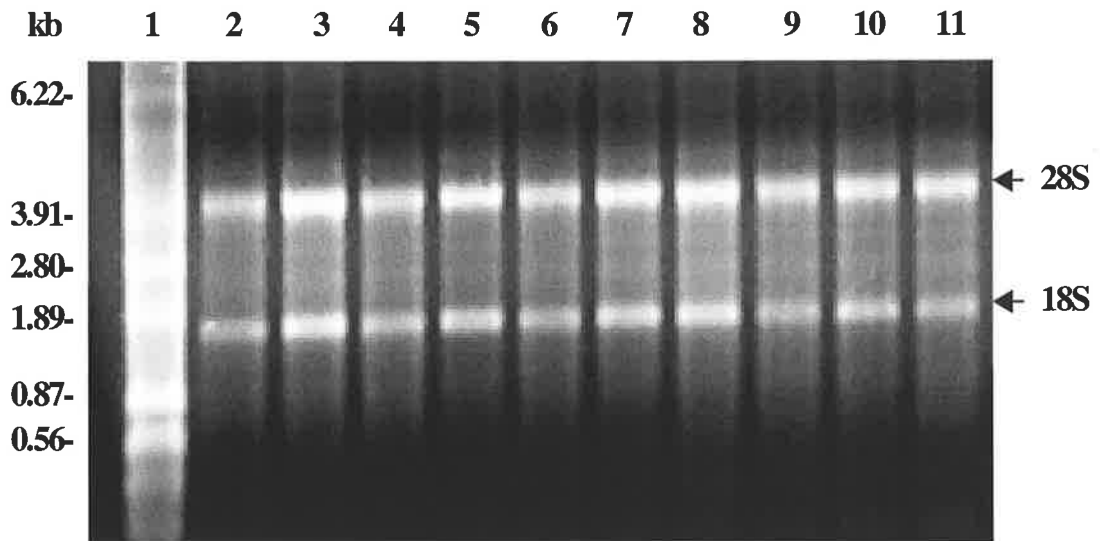


Figure 3.7 Agarose gel electrophoresis of total RNA from a representative number of guinea pig livers

Agarose gel electrophoresis of 1 μg of total RNA from guinea pig livers. RNA from feed restricted pregnant animals are in lanes 2, 6 and 7, feed restricted nonpregnant animals are in lanes 3, 4 and 5, *ad libitum* fed pregnant animals are in lanes 8 and 9 and *ad libitum* fed nonpregnant guinea pigs are in lanes 10 and 11. Lane 1 contains 3 μg of RNA standard (RNA marker, Amersham).

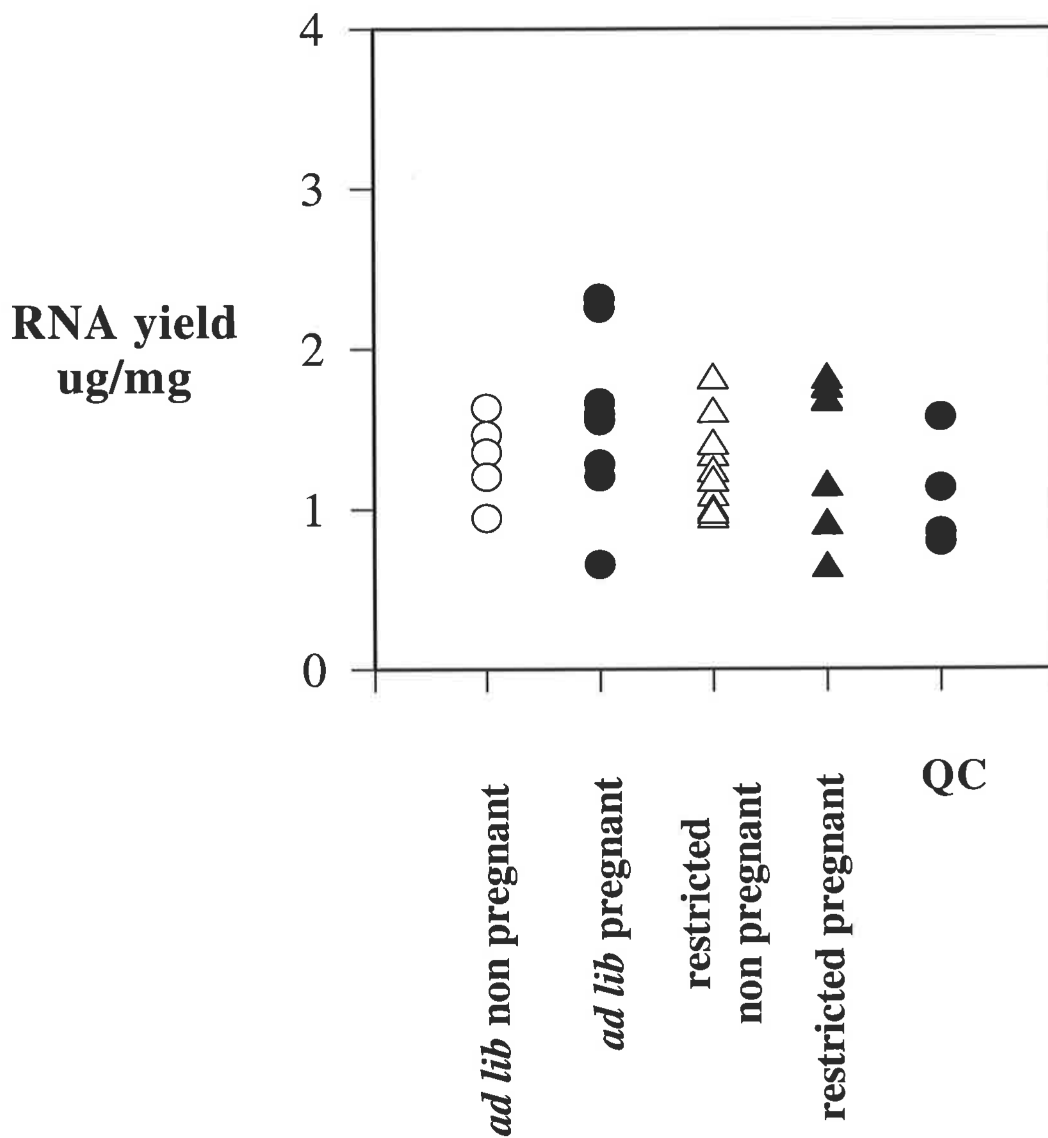


Figure 3.8 RNA yield from guinea pig livers.
Quality control (QC) represents RNA extracted on each day of four days from the same liver specimen (3.2.4).

3.3.6 Effect of pregnancy and nutrition on the relative abundance of IGF-I mRNA

Duplicate analyses of IGF-I mRNA and β-actin mRNA, which included reverse transcription, PCR and Dig-ELISA, were highly correlated (IGF-I, $r = 0.77$, $p<0.001$, β-actin, $r = 0.86$, $p<0.001$) (Figure 3.9). Therefore the average of the duplicate analyses were used in the quantitative estimation of IGF-I and β-actin PCR products.

The relative abundance of IGF-I mRNA, expressed as the ratio of IGF-I to β-actin RT-PCR products in $cDNA_1$ and $cDNA_2$ was highest in *ad libitum* fed pregnant guinea pigs (Figure 3.10). Pregnancy and food availability significantly affected hepatic expression of IGF-I (Table 3.7) whether this was expressed as relative abundance or total abundance (ie relative abundance multiplied by liver weight or fractional liver weight). Pregnancy had a greater effect than nutrition on abundance of hepatic IGF-I mRNA. Relative abundance of hepatic IGF-I mRNA was almost 6-fold higher in pregnant *ad libitum* fed guinea pigs than in nonpregnant *ad libitum* fed guinea pigs.

3.3.7 Relationship between maternal plasma IGF-I and relative abundance of hepatic IGF-I mRNA

Plasma IGF-I was positively correlated with the relative and total abundance of IGF-I mRNA in liver in pregnant and *ad libitum* fed, but not feed restricted guinea pigs (Table 3.8). Polynomial regression analysis showed the overall relationship between the concentration of IGF-I in plasma and the relative abundance of IGF-I mRNA in liver ($r^2 = 0.68$, $p<0.002$) to be second order, $y = 105 + 65x - 1.4x^2$ (Figure 3.11).

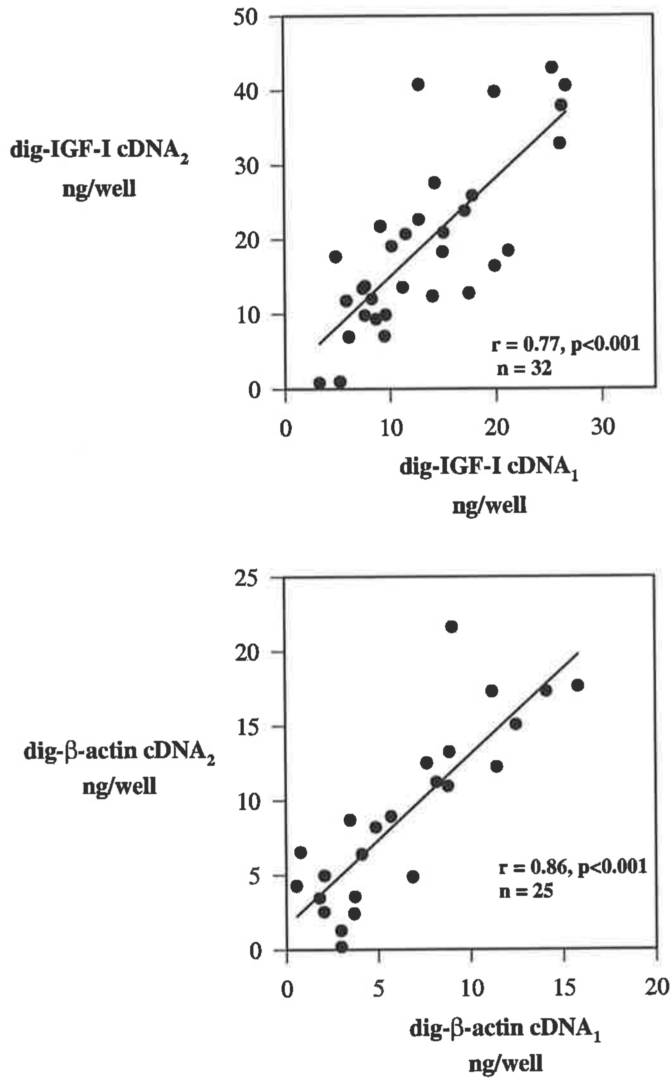


Figure 3.9 Repeatability of Dig-ELISA RT-PCR measurements of guinea pig hepatic RNA

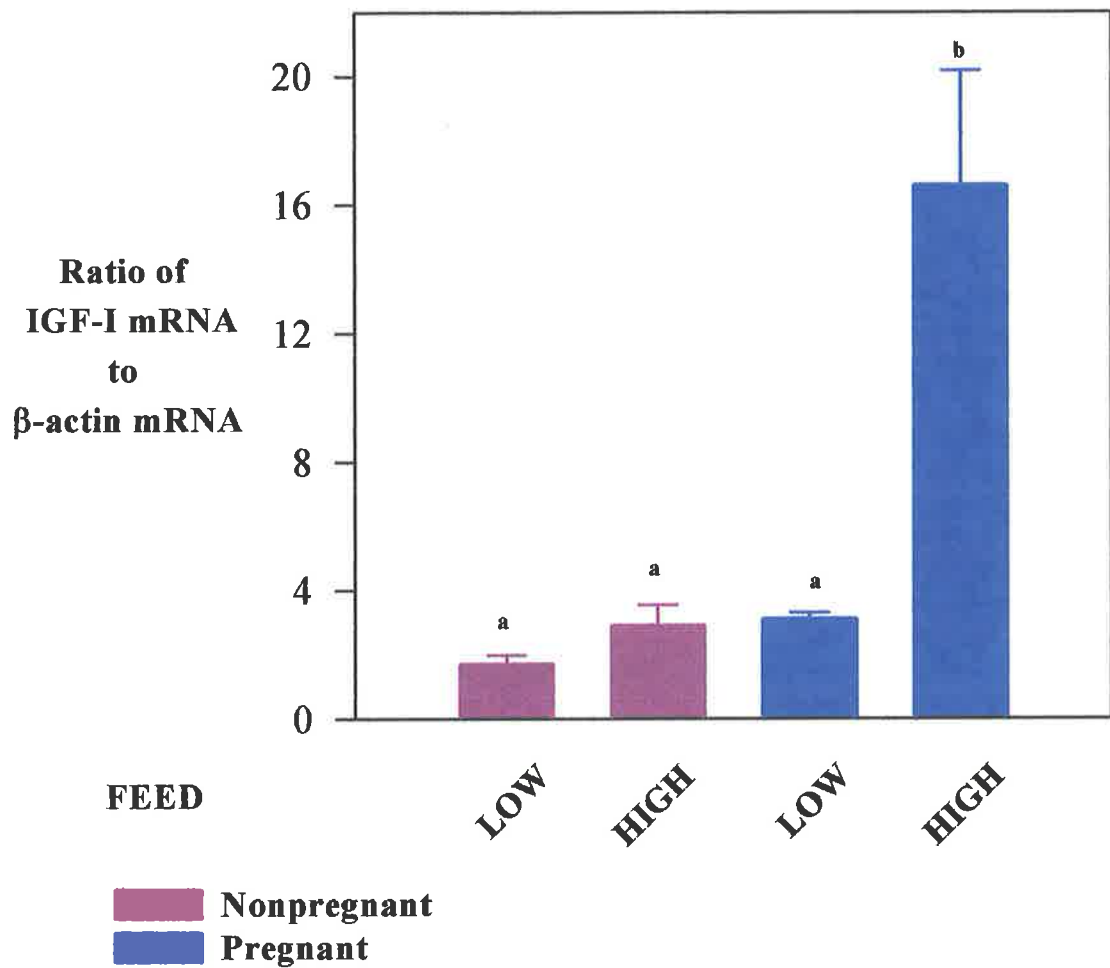


Figure 3.10 Relative abundance of hepatic IGF-I mRNA.
Animals were provided unrestricted (HIGH) or restricted (LOW) access to feed. Different letters depict two-way ANOVA results $p<0.001$.

	Non Pregnant		Pregnant		
	Ad libitum n = 5	Restricted n = 10	*Ad libitum* n = 8	Restricted n = 8	p
IGF-I mRNA/β-actin	2.9 ± 0.7	1.6 ± 0.3	16.6 ± 3.6	3.1 ± 0.23	**F** p <0.001, **P** p<0.001
IGF-I mRNA/β-actin x liver weight	102 ± 29	35.4 ± 7.1	506.5 ± 105	69.7 ± 7.3	**F** p <0.001, **P** p<0.001
IGF-I mRNA/β-actin x liver weight as % body weight	0.14 ± 0.03	0.07 ± 0.01	0.53 ± 0.1	0.1 ± 0.01	**F** p <0.001, **P** p<0.005

Table 3.7 Effects of pregnancy and feed availability on the relative abundance of IGF-I mRNA. Results expressed as mean ± sem. Effect of treatment was assessed by two-way ANOVA with pregnancy status and nutritional regimen as between factors. P= pregnancy, F = feed.

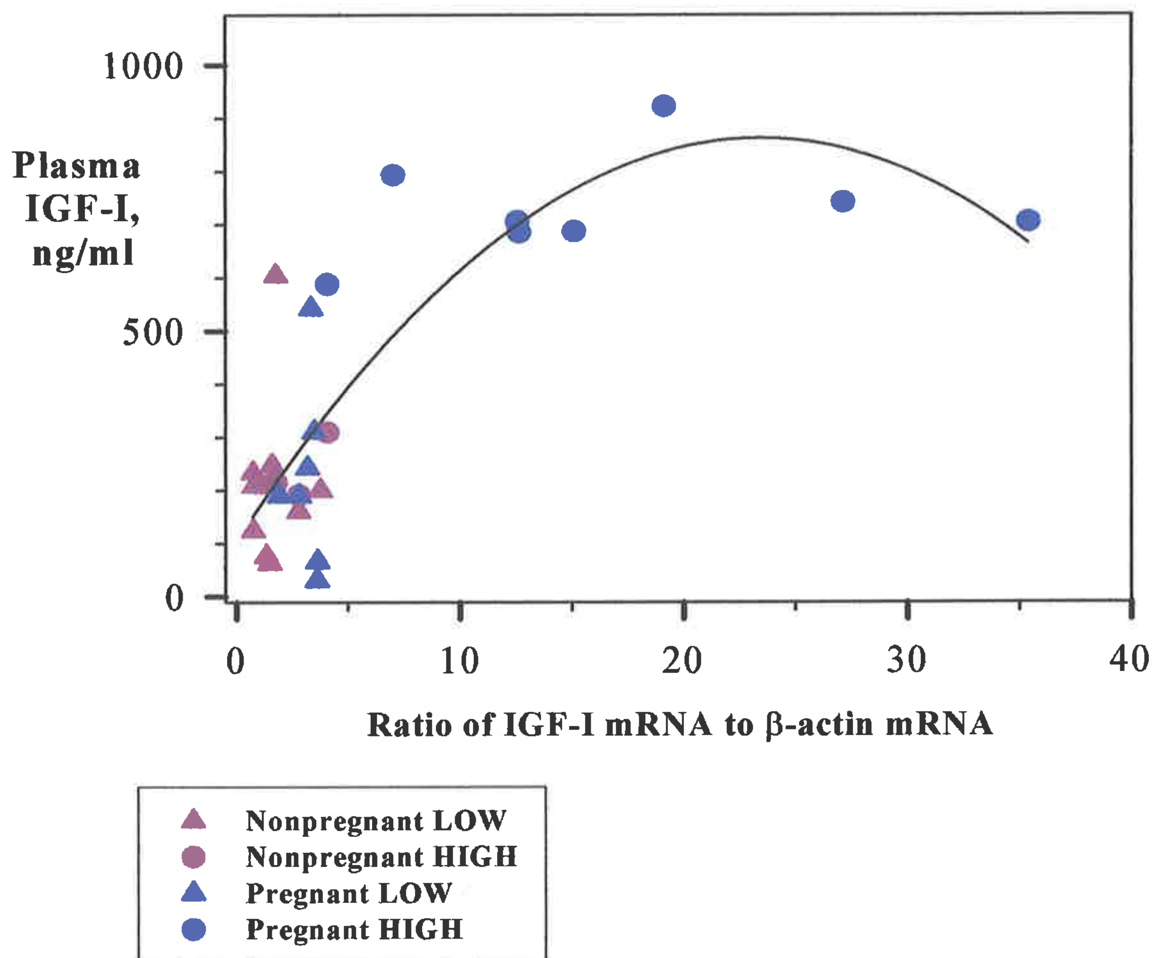


Figure 3.11 Correlation between plasma IGF-I concentration and relative abundance of hepatic IGF-I mRNA.
Plasma IGF-I was positivelly correlated ($r^2 = 0.68$, $p<0.002$) with the relative abundance of hepatic IGF-I mRNA.
The equation of the relationship is $y = 105 + 65x - 1.4x^2$

3.3.8 Relationship between the relative abundance of hepatic IGF-I mRNA and tissue weights

Table 3.9 and Table 3.10 shows the Pearson correlation coefficients between weights of tissues and the relative abundance of hepatic IGF-I mRNA. Combining data from all treatment groups (n = 28) the relative abundance of IGF-I mRNA in liver was positively correlated with body weight, body weight gain and uterine weight, both as a % of body weight and carcass weight. Relative abundance of IGF-I mRNA was also positively correlated with net body weight, net weight gain and weight of carcass and liver.

In pregnant animals (n = 15) the relative abundance of IGF-I mRNA was positively correlated with body weight, weight gain, net weight gain, net body weight and uterine weight both as a % of body weight and carcass weight (Table 3.9, 3.10, Figure 3.12). Relative abundance of hepatic IGF-I mRNA did not correlate with carcass weight or liver weight in pregnant, *ad libitum* fed or feed restricted guinea pigs. In nonpregnant guinea pigs, liver IGF-I mRNA correlated with liver weight and liver as a % of carcass weight, but was not related to carcass weight.

In all *ad libitum* fed animals, hepatic expression of IGF-I was positively correlated with weight gain (Table 3.9), uterine weight, and uterine weight as a % of body weight or as a % of carcass weight (Table 3.10). IGF-I mRNA in feed restricted guinea pigs was positively correlated with weight gain, uterine weight and uterine weight as a % of body weight or as a % of carcass weight. The relative abundance of IGF-I mRNA in liver of *ad libitum* fed and feed restricted guinea pigs was not significantly correlated with absolute weight of liver (Table 3.10).

Weights of retroperitoneal fat, dorsal fat, spleen and soleus muscle were positively correlated overall (n = 28) to the relative abundance of hepatic IGF-I mRNA (Table 3.11).

In pregnant animals (n = 15) the weights of retroperitoneal fat, dorsal fat, spleen and thymus were positively associated with relative abundance of hepatic IGF-I mRNA (Table 3.11). Total gastrointestinal tract weight and soleus muscle weight, were not related to hepatic IGF-I mRNA in pregnant dams. Hepatic IGF-I mRNA was not related to weight of any tissues in the nonpregnant animals (n = 13). Relative abundance of IGF-I mRNA was negatively correlated with retroperitoneal fat weight in *ad libitum* fed guinea pigs (n = 11). In nutritionally restricted animals (n = 17) hepatic IGF-I was negatively correlated with thymus weight only.

3.3.9 Relationship between plasma IGF-I and IGF-II and tissue weights.

Table 3.12, Table 3.13 and Table 3.14 show the Pearson correlation coefficients between weights of maternal tissues and the concentrations of plasma IGF-I. For all data combined (n = 31), plasma IGF-I was positively correlated with body weight, net body weight, weight gain, net weight gain and carcass weight. Plasma IGF-I was positively correlated with uterine weight and uterine weight as a % of body weight and as a % of carcass weight, liver weight, retroperitoneal fat weight, dorsal fat weight, spleen weight and soleus muscle weight. In pregnant and *ad libitum* fed animals plasma IGF-I was positively correlated with body weight, weight gain, uterine weight (Figure 3.12), uterine weight as a % of body weight and as a % of carcass weight, soleus muscle weight and thymus weight.

In pregnant animals, maternal IGF-I was positively correlated with net body weight, net weight gain, carcass weight, liver weight and liver weight as a % of body weight and was

Plasma IGF-I ng/ml

	Overall n = 28	**Pregnant n = 15**	**Non pregnant n= 13**	***Ad libitum* Fed n = 11**	**Restricted n = 17**
IGF-I mRNA to β-actin	*0.71 p <0.0001*	*0.65 p < 0.01*	*0.16 NS*	*0.62 p = 0.04*	*0.05 NS*
IGF-I mRNA to β-actin * Liver wt	*0.74 p < 0.0001*	*0.7 p < 0.01*	*0.23 NS*	*0.65 p = 0.02*	*0.15 NS*
IGF-I mRNA to β-actin* Liver wt/body weight	*0.71 p < 0.0001*	*0.67 p <0.01*	*0.17 NS*	*0.61 p = 0.045*	*0.05 NS*

Table 3.8 Associations between plasma IGF-I and abundance of hepatic IGF-I mRNA.

Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

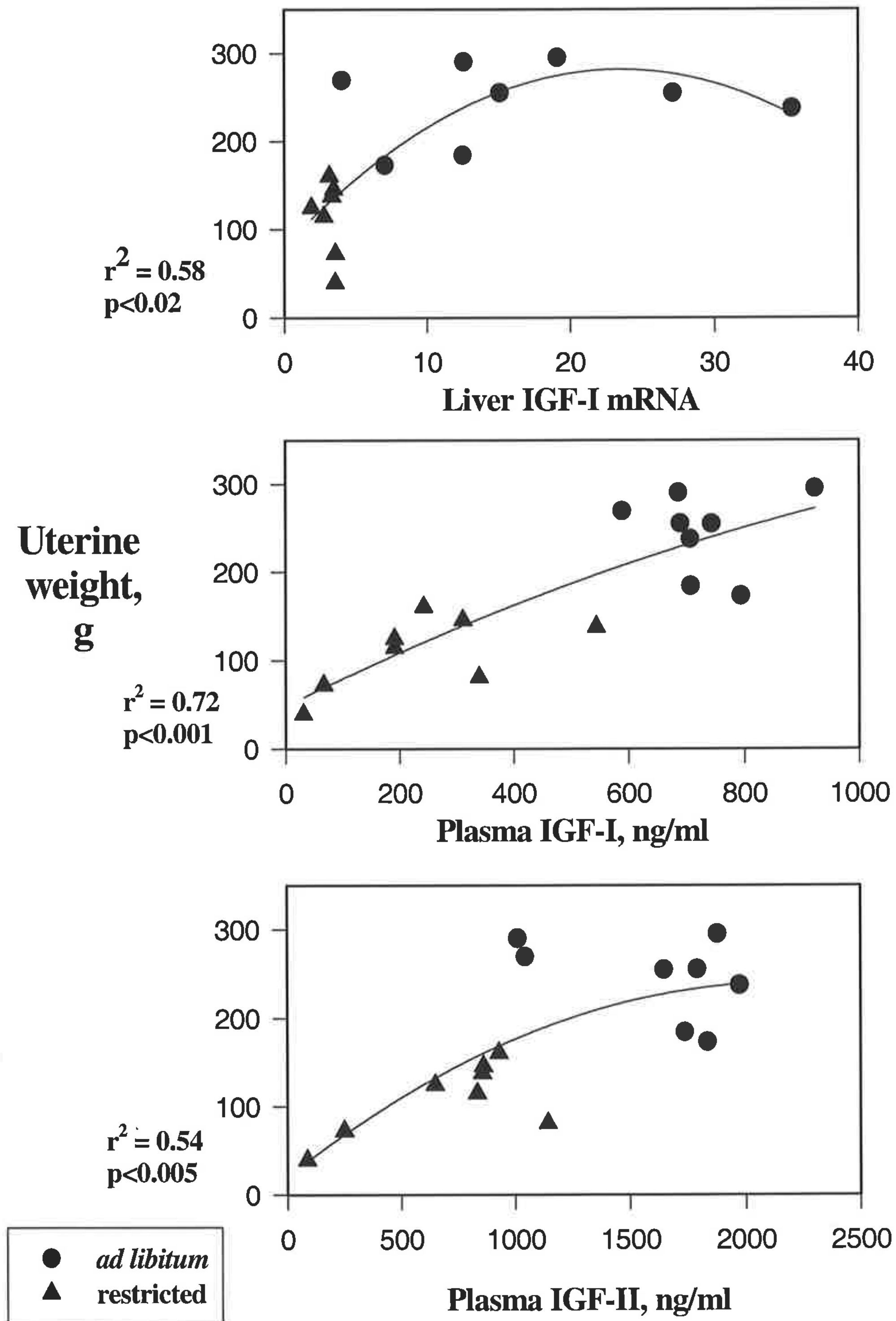


Figure 3.12 Effect of nutrition on the relationships between uterine weight hepatic abundance of IGF-I mRNA, endocrine IGF-I and IGF-II

Relative abundance of IGF-I mRNA

	Overall n = 28	**Pregnant** n = 15	**Non pregnant** n =13	***Ad libitum* fed** n = 11	**Restricted** n = 17
Body weight	0.68, $p <0.0001$	0.61, $p = 0.01$	*0.52, NS*	0.46, *NS*	0.54, $p = 0.02$
Net body weight	0.48, $p < 0.01$	0.54, $p = 0.03$	0.51, *NS*	-0.38, *NS*	*0.28, NS*
Weight gain	*0.74, p < 0.0001*	*0.69, p < 0.01*	*0.52, NS*	0.61, $p = 0.04$	*0.64, p <0.01*
Net weight gain	*0.47, p = 0.01*	*0.58, p = 0.02*	*0.52, NS*	*-0.34, NS*	*0.22, NS*
Carcass weight	0.47, $p= 0.01$	0.49, *NS*	*0.36, NS*	*-0.37, NS*	*0.06, NS*
Carcass as % of body weight	*0.23, NS*	0.29, *NS*	*-0.43, NS*	*-0.12, NS*	*-0.37,NS*

Table 3.9 Associations between the relative abundance of hepatic IGF-I mRNA with body phenotype. Pearson correlation coefficients r are tabulated and p is significant at p<0.05. Pregnant and nonpregnant groups include both *ad libitum* fed and feed restricted animals. The *ad libitum* fed and restricted groups include both pregnant and nonpregnant animals.

Relative abundance of IGF-I mRNA

Tissue weight (g)	Overall n = 28	Pregnant n = 15	Non pregnant n =13	*Ad libitum* fed n = 11	Restricted n = 17
Uterus, g	0.7, $p < 0.0001$	0.62, $p = 0.01$	*0.18, NS*	*0.6,* $p = 0.04$	*0.57,* $p = 0.01$
Uterus as % of body weight	0.67, $p < 0.0001$	*0.56,* $p = 0.02$	-0.23, *NS*	*0.63,* $p = 0.03$	*0.56,* $p = 0.01$
Uterus as % of carcass	*0.65,* $p < 0.0001$	*0.52,* $p = 0.04$	*-0.12, NS*	*0.65,* $p = 0.02$	*0.56,* $p = 0.01$
Liver, g	*0.4,* $p = 0.03$	*0.49, NS*	*0.56,* $p = 0.04$	*-0.57, NS*	*0.23, NS*
Liver as % of body weight	*0.02, NS*	*0.16, NS*	*0.42, NS*	*-0.55, NS*	*0.004, NS*
Liver as % of carcass weight	*-0.13, NS*	*-0.06, NS*	*0.63,* $p = 0.02$	*-0.34, NS*	*0.24, NS*

Table 3.10 Associations between relative abundance of hepatic IGF-I mRNA with tissue weights and plasma IGF-I. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Relative abundance of IGF-I mRNA

Tissue weight (g)	**Overall n = 28**	**Pregnant n = 15**	**Non pregnant n =13**	***Ad libitum* fed n = 11**	**Restricted n = 17**
Retroperitoneal fat	*0.45, p<0.02*	*0.52, p<0.05*	0.45, *NS*	*-0.64, p<0.05*	*0.06, NS*
Dorsal fat	*0.48, p<0.01*	*0.55, p<0.05*	*0.48, NS*	*-0.37, NS*	*0.2, NS*
Total gastrointestinal tract	*-0.25, NS*	*-0.3, NS*	*-0.29, NS*	*-0.05, NS*	*0.22, NS*
Spleen	*0.6, p<0.001*	*0.74, p<0.002*	*0.47, NS*	*0.22, NS*	*-0.11, NS*
Thymus	*0.29, NS*	*0.66, p<0.01*	*0.39, NS*	*-0.45, NS*	*-0.49, p<0.05*
Soleus muscle	*0.57, p<0.002*	*0.5, NS*	*0.45, NS*	*0.14, NS*	*0.23, NS*

Table 3.11 Associations between the relative abundance of hepatic IGF-I mRNA and weights of tissues. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Plasma IGF-I ng/ml

	Overall n = 31	Pregnant n = 16	Non pregnant n =15	*Ad libitum* fed n = 13	Restricted n = 18
Body weight	*0.82, p < 0.0001*	*0.87, p < 0.0001*	*0.44, NS*	*0.84, p < 0.001*	*0.4, NS*
Net body weight	*0.59, p < 0.0001*	*0.78, p< 0.001*	*0.44, NS*	*-0.27, NS*	*0.39, NS*
Weight gain	*0.82, p<0.0001*	*0.87, p<0.0001*	*0.35,NS*	*0.87, p < 0.0001*	*0.26, NS*
Net weight gain	*0.45, p = 0.01*	*0.67, p < 0.01*	*0.35, NS*	*-0.51, NS*	*0.06, NS*
Carcass weight	*0.6, p < 0.001*	*0.74, p =.001*	*0.46, NS*	*-0.13, NS*	*0.31, NS*
Carcass as % of body weight	*0.32, NS*	*0.4, NS*	*0.24, NS*	*0.17, NS*	*-0.14, NS*

Table 3.12 Associations between plasma IGF-I and adult body phenotype. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Plasma IGF-I ng/ml

Tissue weight (g)	Overall n = 31	Pregnant n = 16	Non pregnant n =15	*Ad libitum* fed n = 13	Restricted n = 18
Uterus, g	*0.78, p < 0.0001*	*0.84, p < 0.0001*	*0.4, NS*	*0.91, p < 0.0001*	*0.26, NS*
Uterus as % of body weight	*0.72, p < 0.0001*	*0.75, p < 0.001*	*0.12, NS*	*0.9, p < 0.0001*	*0.27, NS*
Uterus as % of carcass weight	*0.7, p<0.0001*	*0.74, p<0.01*	*0.07, NS*	*0.91, p<0.0001*	*0.26, NS*
Liver, g	*0.52, p < 0.01*	*0.83, p < 0.0001*	*0.39, NS*	*-0.48, NS*	*0.49, p = 0.03*
Liver as % of body weight	*0.18, NS*	*0.49, p = 0.04*	*0.18, NS*	*-0.56, p = 0.04*	*0.25, NS*
Liver as % of carcass weight	*-0.06, NS*	*0.15, NS*	*-0.01, NS*	*-0.49, NS*	*0.29, NS*

Table 3.13 Associations between plasma IGF-I and adult tissue weights. Pearson correlation coefficients r are tabulated and p is significant at p<0.05.

Plasma IGF-I ng/ml

Tissue weight (g)	Overall n = 31	Pregnant n = 16	Non pregnant n =15	*Ad libitum* fed n = 13	Restricted n = 18
Retroperitoneal fat	*0.56, p<0.002*	*0.84, p<0.0001*	*0.21, NS*	*-0.37, NS*	*0.01, NS*
Dorsal fat	*0.54, p<0.002*	*0.81, p<0.0001*	*0.26, NS*	*-0.3, NS*	*0.07, NS*
Total gastrointestinal tract	*-0.29, NS*	*-0.57, p<0.05*	*0.42, NS*	*-0.56, NS*	*0.31, NS*
Spleen	*0.56, p<0.001*	*0.82, p<0.0001*	*0.32, NS*	*-0.03, NS*	*0.08, NS*
Thymus	*0.33, NS*	*0.87, p<0.0001*	*0.42, NS*	*-0.73, p<0.005*	*0.26, NS*
Soleus muscle	*0.73, p<0.0001*	*0.78, p<0.0001*	*0.37, NS*	*0.55, p<0.05*	*0.32, NS*

Table 3.14 Associations between plasma IGF-I and adult weights tissues. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

negatively correlated with total gastrointestinal tract weight. In *ad libitum* fed animals, liver as a % of body weight and thymus were negatively correlated with plasma IGF-I. In feed restricted animals only liver weight was positively correlated with circulating levels of IGF-I.

Tables 3.15, 3.16 and 3.17 shows the Pearson correlation coefficients between weights of maternal tissues and the concentrations of plasma IGF-II. For all data combined (n =31), plasma IGF-II was highly positively correlated with body weight, net body weight, weight gain, net weight gain and carcass weight (Table 3.15).

Plasma IGF-II was positively correlated with uterine weight, liver and with the weights of retroperitoneal fat, dorsal fat, spleen, soleus muscle and thymus (Table 3.16, 3.17). In pregnant and nonpregnant animals circulating IGF-II was positively correlated with body weight, net body weight, weight gain, net weight gain and carcass weight, uterine weight and liver weight (Table 3.15, 3.16). Plasma IGF-II was positively correlated with the weights of retroperitoneal fat, dorsal fat, spleen, soleus muscle and thymus (Table 3.17). In pregnant animals, plasma IGF-II was positively correlated with uterine weight as a % of body weight and negatively related to total gastrointestinal tract weight. In nonpregnant animals liver as a % of body weight was correlated with plasma IGF-II. Plasma IGF-II was not related to maternal body weights or tissue weights in *ad libitum* fed or feed restricted animals.

Plasma IGF-II ng/ml

	Overall n = 31	Pregnant n = 16	Non pregnant n =15	*Ad libitum* fed n = 13	Restricted n = 18
Body weight	*0.72, p < 0.0001*	*0.78, p < 0.0001*	*0.92, p<0.0001*	*-0.009, NS*	*0.29, NS*
Net body weight	*0.79, p < 0.0001*	*0.75, p< 0.001*	*0.92, p<0.0001*	*0.06, NS*	*0.39, NS*
Weight gain	*0.67, p<0.0001*	*0.74, p<0.002*	*0.89, p<0.0001*	*0.02, NS*	*-0.02, NS*
Net weight gain	*0.7, p< 0.001*	*0.75, p< 0.001*	*0.92, p<0.0001*	*0.15, NS*	*-0.18, NS*
Carcass weight	*0.77, p < 0.0001*	0.68, *p<0.005*	*0.9, p<0.0001*	*-0.07, NS*	*0.23, NS*
Carcass as % of body weight	*0.3, NS*	*0.33, NS*	*0.24, NS*	*0.18, NS*	*-0.28, NS*

Table 3.15 Relationship between plasma IGF-II and adult body phenotype. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Plasma IGF-II ng/ml

Tissue weight (g)	Overall n = 31	Pregnant n = 16	Non pregnant n =15	*Ad libitum* fed n = 13	Restricted n = 18
Uterus, g	*0.4, p < 0.05*	*0.71, p < 0.005*	*0.64, p<0.01*	*-0.03, NS*	*0.08, NS*
Uterus as % of body weight	*0.33, NS*	*0.6, p < 0.02*	*0.009, NS*	*-0.04, NS*	*0.08, NS*
Uterus as % of carcass	*0.3, NS*	*0.57, NS*	*-0.03, NS*	*-0.02, NS*	*0.09, NS*
Liver, g	*0.76, p < 0.0001*	*0.73, p < 0.002*	*0.88, p<0.0001*	*-0.01, NS*	*0.4, p = 0.03*
Liver as % of body weight	*0.34, NS*	*0.3, NS*	*0.54, p<0.05*	*-0.03, NS*	*0.1, NS*
Liver as % of carcass weight	*0.09, NS*	*0.02, NS*	*0.28, NS*	*-0.01, NS*	*0.25, NS*

Table 3.16 Relationship between plasma IGF-II and adult body composition. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Plasma IGF-II ng/ml

Tissue weight (g)	**Overall n = 31**	**Pregnant n = 16**	**Non pregnant n =15**	***Ad libitum* fed n = 13**	**Restricted n = 18**
Retroperitoneal fat	*0.8, p<0.0001*	*0.77, p<0.0001*	0.86, p<0.001	*0.08, NS*	*0.0, NS*
Dorsal fat	*0.8, p<0.0001*	*0.77, p<0.0001*	*0.88, p<0.0001*	*0.28, NS*	*0.09. NS*
Total gastrointestinal tract	*-0.29, NS*	*-0.55, p<0.05*	*0.33,NS*	*-0.06, NS*	*0.05, NS*
Spleen	*0.82, p<0.0001*	*0.8, p<0.0001*	*0.88, p<0.0001*	*0.3, NS*	*0.37, NS*
Thymus	*0.75, p<0.0001*	*0.85, p<0.0001*	*0.89, p<0.0001*	*0.23, NS*	*0.36, NS*
Soleus muscle	*0.76, p<0.0001*	*0.82, p<0.0001*	*0.79, p<0.0001*	*0.48, NS*	*0.14, NS*

Table 3.17 Relationship between plasma IGF-II and adult tissue weights. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Tables 3.18, 3.19 and 3.20 show the results of analysis of components of the endocrine IGF system as covariates (independent variable) of tissue weights (dependent variable). Forward stepwise multiple regression analysis was used to determine whether any combination of IGF endocrine variables were covariates of the weights of organs or tissues.

The effect of nutrition in nonpregnant animals on weights of most tissues was dependent on the concentration of IGF-II in plasma, except for carcass as a fraction of body weight where IGFBP-2 was the dominant independent covariate and retroperitoneal fat and soleus muscle, where IGFBP-3 was the dominant independent variable (Table 3.18, 3.19). Pregnancy abolishes these nutritionally determined relationships except in the case of carcass as a % of body weight and retroperitoneal fat. In the case of uterine weight IGF-II was the major nutritionally determined correlate in nonpregnant animals whereas IGF-I was the major nutritionally determined correlate in pregnancy (Table 3.19). In pregnant animals, IGF-I was the major nutritionally determined correlate for other tissue weights, including body weight, liver weight and total gastrointestinal tract weight, with the exception of soleus muscle where IGF-II dominates (Table 3.18, 3.19).

The concentration of IGFBP-3 in plasma was the major independent covariate of the effect of nutrition in pregnant animals on net body weight, weight gain and weights of carcass, retroperitoneal fat, dorsal fat, spleen and thymus while its effect on net weight gain in pregnancy was related to IGFBP-2 (Table 3.18, 3.19). In pregnant *ad libitum* fed animals, body weight, weight gain, uterine weight and thymic weight were related to plasma IGF-I, whereas, IGF-I and IGFBP-1 were the best covariates of soleus muscle weight. There were no independent covariates for any of the other tissues in pregnant animals.

In pregnant underfed animals, tissue weights were related to the plasma concentrations of either IGFBP-2 or IGFBP-1 except for liver where IGF-I was the best independent covariate (Table 3.20). In feed restricted animals, net weight gain and carcass weights were related to IGFBP-3 (Table 3.18).

In *ad libitum* fed animals, no independent covariates were identified for retroperitoneal or dorsal fat. In all undernourished animals circulating concentrations of IGF-binding proteins are major correlates of tissue weights except for liver where IGF-I is the major independent covariate (Table 3.20).

The effect of nutrition in pregnancy on the weights of many tissues was dependent on the ratio of the concentration of IGF-I to that of IGFBP-1 in plasma (Table 3.21, 3.22). The exceptions were body weight, weight gain, carcass weight, carcass weight as a % of body weight as well as uterine, retroperitoneal fat and total gastrointestinal tract weight, where the ratio of the concentration of IGF-I to the concentration of IGFBP-2 in plasma were the dominant independent covariates.

In nonpregnant animals tissue weights were related to the ratio of the concentration of IGF-II to the concentration of IGFBP-2 in plasma. Uterine weight where the ratio of the concentration of IGF-II to that of IGFBP-4 was the major nutritionally determined correlate, and total gastrointestinal tract weight where the ratio of IGF-II to IGFBP-1 concentrations was the major nutritionally dependent correlate were the exceptions (Table 3.21, 3.22). In nonpregnant animals all significant correlations contained plasma IGF-II concentrations as a covariate whereas in pregnancy almost all significant relationships contained plasma IGF-I concentration except for net weight gain for which concentration of plasma IGF-II was an independent covariate.

In *ad libitum* fed guinea pigs body weight, weight gain, uterine and thymus weight were dependent on the ratio of the concentration of IGF-I to those of IGFBP-3 in plasma whereas soleus muscle weight was related to the ratio of IGF-I to IGFBP-1 (Table 3.23, 3.24). In nutritionally restricted animals, tissue weights were determined by the ratio of plasma IGF-II to IGFBP-1 concentrations except for net weight gain, which was related to the ratio of IGF-II to IGFBP-3 concentrations (Table 3.23, 3.24). Thymus weight was determined by the ratio of plasma IGF-II to IGFBP-2 concentrations in the feed restricted animals (Table 3.24). In undernourished animals all of the IGF to IGFBP ratios which were related to tissue weights contained plasma IGF-II and none contained IGF-I concentration.

3.3.10 Relationship between maternal IGF endocrine axis and fetal and placental weights in pregnant guinea pigs

Relative abundance of hepatic IGF-I to β-actin ratio

The relative abundance of maternal hepatic IGF-I mRNA was positively correlated with total fetal weight, total placental weight, average fetal weight and the ratio of average fetal to placental weight (Table 3.25). Total fetal weights, both as a % of maternal body weight and as a % of maternal carcass weight, were significantly associated with maternal hepatic IGF-I mRNA relative abundance.

Plasma IGFs

Maternal plasma IGF-I concentration was positively correlated with number of fetuses per dam, total fetal weight, total fetal weight as a % of body weight or carcass weight, total placental weight and total placental weight expressed as % of body weight or carcass weight, average fetal weight and average placental weight (Table 3.25).

Maternal plasma IGF-II was positively correlated with average fetal weight, total fetal weight, the ratio of average fetal to placental weight, total placental weight, total fetal weight as a % of body weight or carcass weight, and average placental weight and total placental weight expressed as % of body weight (Table 3.25).

Insulin-like growth factor binding proteins

The concentrations of IGFBP-1 and IGFBP-4 in maternal plasma were not significantly related to any of the fetal and placental variables measured (data not shown). Maternal plasma concentrations of IGFBP-3 were positively correlated with total fetal weight and total fetal weight as a % of body weight, total placental weight, average fetal weight, average placental weight and average fetal to placental weight ratio (Table 3.25). Maternal plasma concentrations of IGFBP-2 were negatively correlated with total fetal weight, total placental weight, average fetal weight and average placental weight.

Table 3.27 shows the covariate analyses of determinants of fetal and placental weights (dependent variables) amongst components of the IGF system (independent variable). Forward stepwise multiple linear regression analysis was performed with six independent variables included to determine whether the IGF endocrine variables were predictors of fetal and/or placental growth.

The concentration of IGF-I in maternal plasma was an independent covariate of the number of fetuses per dam, total fetal weight, total fetal weight as a % of body weight or carcass weight, total placental weight, total placental weight as a % of body weight or carcass weight and the ratio of average fetal to placental weight (Table 3.27). Average fetal weight was dependent on plasma IGFBP-3 and average placental weight was dependent on plasma IGFBP-2 (Table 3.27). In pregnant feed restricted animals number of fetuses per dam was determined by maternal plasma IGF-I but total placental weight was determined by maternal plasma IGF-II.

There were no independent covariates for any other fetal or placental measures in nutritionally restricted or *ad libitum* fed pregnant animals.

Ratios of insulin-like growth factors to insulin-like growth factor binding proteins

The ratios of the concentrations of IGFs to IGFBPs in plasma were assessed as independent covariates of maternal and fetal tissues to identify potential IGF/IGFBP complexes as determinants. The ratio of the concentration of IGF-I to IGFBP-3 in maternal plasma was positively associated with uterine weight as a % of carcass weight, number of fetuses per dam, total fetal weight as a % of body weight or carcass weight and total placental weight as a % of body weight or carcass weight (Table 3.26).

The ratio of the concentrations of IGF-I to IGFBP-1 in plasma was a positive independent covariate of all maternal and fetal tissue weights (Table 3.26) except for number of fetuses per dam and total placental weight as a % of body weight or carcass weight. Ratio of plasma IGF-I to IGFBP-2 concentrations was a positive independent covariate of uterine weight, uterine weight as a % of body weight or carcass weight, total fetal weight, total fetal weight as a % of body weight or carcass weight, total placental weight as a % of body weight, average fetal weight and average placental weight. The ratio of the concentrations of IGF-I to IGFBP-4 in plasma was a positive independent covariate of uterine weight, total fetal weight, total placental weight and average fetal weight.

The ratio of plasma IGF-II to IGFBP-3 concentrations and the ratio of IGF-II to IGFBP-4 concentrations were not associated with any maternal or fetal weights (data not shown). The ratio of plasma IGF-II to IGFBP-1 and the ratio of IGF-II to IGFBP-2 concentrations were positive independent covariates of uterine weight, total fetal weight, total placental weight, average fetal weight and average placental weight. The ratio of the concentrations of IGF-II to

those of IGFBP-2 in plasma was a positive independent covariate of uterine weight as a % of body weight and total fetal weight as a % of body weight.

Table 3.28 shows the covariate analyses of the dependence of fetal and placental parameters (dependent variable) on the ratio of the concentrations of IGF-Is in plasma to those of IGFBPs (independent variable). Forward stepwise regression included eight independent variables.

All fetal and placental weights were independently determined by the ratio of the concentrations of IGF-I to those of IGFBP-2 in plasma (Table 3.28, Figure 3.13) except for the ratio of average fetal to placental weight, which was determined by the ratio of plasma IGF-I to IGFBP-1 concentrations, although this was a weak association. In feed restricted pregnant guinea pigs total fetal and placental weights and fetuses per dam were strongly dependent on the ratio of plasma IGF-I to IGFBP-1concentrations (Table 3.29). None of these above relationships contained IGF-II as a significant independent covariate. In *ad libitum* fed pregnant animals total fetal weight and number of fetuses per dam were dependent on the ratio of the concentrations of IGF-II to IGFBP-4 in plasma.

Dependent Variable	Pregnant	Nonpregnant	*Ad-libitum* fed	Restricted
Body weight	IGF-I +, $\delta r^2 = 0.75, p<0.01$ IGFBP-3 +, $\delta r^2 = 0.07, p<0.05$	IGF-II +, $\delta r^2 = 0.85, p<0.01$	IGF-I +, $\delta r^2 = 0.72, p<0.001$	IGFBP-1 +, $\delta r^2 = 0.49, p<0.001$ IGF-II +, $\delta r^2 = 0.2, p<0.01$ IGFBP-2 +, $\delta r^2 = 0.1, p<0.01$
Net Body weight	IGFBP-3 + δ, $r^2 = 0.67, p<0.01$ IGF-II +, $\delta r^2 = 0.09, p<0.005$	IGF-II +, $\delta r^2 = 0.85, p<0.01$		IGF-II +, $\delta r^2 = 0.24, p<0.05$ IGFBP-1 +, $\delta r^2 = 0.2, p<0.02$
Weight gain	IGFBP-3 +, $\delta r^2 = 0.77, p<0.01$ IGF-I +, $\delta r^2 = 0.1, p<0.01$	IGF-II +, $\delta r^2 = 0.79, p<0.002$	IGF-I +, $\delta r^2 = 0.77, p<0.001$	IGFBP-1 +, $\delta r^2 = 0.75, p<0.001$
Net weight gain	IGFBP-2 -, $\delta r^2 = 0.67, p<0.01$ IGFBP-3 +, $\delta r^2 = 0.14, p<0.01$	IGF-II +, $\delta r^2 = 0.79, p<0.002$		IGFBP-3 +, $\delta r^2 = 0.35, p<0.01$
Carcass weight	IGFBP-3 +, $\delta r^2 = 0.7, p<0.001$	IGF-II +, $\delta r^2 = 0.83, p<0.02$ IGFBP-3 +, $\delta r^2 = 0.06, p<0.05$		IGFBP-3 +, $\delta r^2 = 0.23, p<0.05$
Carcass as % of body weight	IGFBP-2 +, $\delta r^2 = 0.47, p<0.001$	IGFBP-2 -, $\delta r^2 = 0.32, p<0.05$		IGFBP-2 +, $\delta r^2 = 0.26, p<0.01$ IGFBP-4 +, $\delta r^2 = 0.21, p<0.05$

Table 3.18 Summary of endocrine IGF variables as independent covariates identified as predictors of body phenotype in pregnant, nonpregnant, *ad libitum* fed and feed restricted guinea pigs. A combination of 6 independent IGF endocrine variables were entered into forward stepwise linear regression.

Table 3.19 Summary of endocrine IGF variables as independent covariates identified as predictors of tissue weights in pregnant and nonpregnant guinea pigs. A combination of 6 independent IGF endocrine variables were entered into forward stepwise linear regression.

Dependent variable	Independent Covariates of IGF endocrine factors	
Tissue weight (g)	**Pregnant**	**Nonpregnant**
Uterus, g	IGF-I +, $\delta\ r^2 = 0.71$, $p<0.0001$	IGF-II +, $\delta\ r^2 = 0.41$, $p<0.01$
Retroperitoneal fat, g	IGFBP-3 +, $\delta\ r^2 = 0.7$, $p<0.002$ IGFBP-2 -, $\delta\ r^2 = 0.11$, $p<0.01$ IGFBP-1 -, δ r2 = 0.07, $p<0.02$	IGFBP-3 +, $\delta\ r^2 = 0.8$, $p<0.0001$
Dorsal fat, g	IGFBP-3 +, $\delta\ r^2 = 0.71$, $p<0.001$ IGFBP-1 -, $\delta\ r^2 = 0.1$, $p<0.005$ IGFBP-2 -, δ r2 = 0.08, $p<0.01$	IGF-II +, $\delta\ r^2 = 0.78$, $p<0.0001$
Total gastrointestinal tract, g	IGF-I -, $\delta\ r^2 = 0.32$, $p<0.005$ IGFBP-4 -, $\delta\ r^2 = 0.18$, $p=0.05$	
Spleen, g	IGFBP-3 +, $\delta\ r^2 = 0.73$, $p<0.001$ IGF-II +, $\delta\ r^2 = 0.13$, $p<0.005$	IGF-II +, $\delta\ r^2 = 0.77$, $p<0.0001$
Thymus, g	IGFBP-3 +, $\delta\ r^2 = 0.78$, $p<0.0001$ IGF-II +, $\delta\ r^2 = 0.15$, $p<0.001$	IGF-II +, $\delta\ r^2 = 0.8$, $p<0.0001$
Soleus, g	IGF-II +, δ r2 = 0.67, $p<0.0001$	IGFBP-3 +, $\delta\ r^2 = 0.71$, $p<0.0001$
Liver, g	IGF-I +, $\delta\ r^2 = 0.69$, $p<0.0001$	IGF-II +, $\delta\ r^2 = 0.78$, $p<0.005$ IGFBP-1 +, $\delta\ r^2 = 0.05$, $p=0.06$

Table 3.20 Summary of endocrine IGF variables as independent covariates identified as predictors of tissue weights in *ad libitum* fed and feed restricted guinea pigs. A combination of 6 independent IGF endocrine variables were entered into forward stepwise linear regression.

Dependent variable	Independent Covariates of IGF endocrine factors	
Tissue weights (g)	***ad libitum* fed, pregnant and nonpregnant**	**Underfed, pregnant and nonpregnant**
Uterus, g	IGF-I +, $\delta r^2 = 0.83$, $p<0.0001$ IGF-II +, $\delta r^2 = 0.09$, $p<0.005$	IGFBP-2 +, $\delta r^2 = 0.57$, $p<0.0002$ IGFBP-1 +, $\delta r^2 = 0.16$, $p<0.02$ IGF-I +, $\delta r^2 = 0.06$, $p<0.05$
Retroperitoneal fat, g		
Dorsal fat, g		
Total gastrointestinal tract, g		IGFBP-1 +, $\delta r^2 = 0.26$, $p<0.05$
Spleen, g		
Thymus, g	IGF-I -, $\delta r^2 = 0.53$, $p<0.0001$ IGF-II +, $\delta r^2 = 0.21$, $p<0.001$ IGFBP-3 +, $\delta r^2 = 0.08$, $p<0.001$ IGFBP-4 -, $\delta r^2 = 0.1$, $p<0.002$	IGFBP-2 -, $\delta r^2 = 0.32$, $p<0.02$
Soleus, g	IGFBP-1 -, $\delta r^2 = 0.31$, $p<0.02$ IGF-I +, $\delta r^2 = 0.27$, $p<0.001$ IGFBP-2 -, $\delta r^2 = 0.13$, $p<0.005$ IGFBP-3 +, $\delta r^2 = 0.12$, $p<0.01$	IGFBP-1 +, $\delta r^2 = 0.40$, $p<0.005$
Liver, g		IGF-I +, $\delta r^2 = 0.24$, $p<0.05$

Table 3.21 Summary of independent covariates identified as predictors of body phenotype in pregnant and nonpregnant guinea pigs. A combination of eight independent variables the ratio of IGF-I, -II to IGFBP-1, -2, -3, -4 were included in the analysis.

Dependent variable	Independent Covariates ratio of IGFs/IGFBPs	
	Pregnant	**Nonpregnant**
Body weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.61$, $p<0.001$	Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.62$, $p<0.001$
Net body weight	Ratio of IGF-I/IGFBP-1 +, $\delta r^2 = 0.58$, $p<0.001$	Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.62$, $p<0.001$
Weight gain	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.66$, $p<0.005$	Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.64$, $p<0.001$ Ratio of IGF-I/IGFBP-1 -, $\delta r^2 = 0.17$, $p<0.01$
Net weight gain	Ratio of IGF-II/IGFBP-3 -, $\delta r^2 = 0.53$, $p<0.001$ Ratio of IGF-II/IGFBP-1 +, $\delta r^2 = 0.25$, $p<0.001$	Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.64$, $p<0.001$ Ratio of IGF-I/IGFBP-1 -, $\delta r^2 = 0.17$, $p<0.01$
Carcass weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.56$, $p<0.001$	Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.64$, $p<0.001$ Ratio of IGF-I/IGFBP-1 -, $\delta r^2 = 0.1$, $p<0.05$
Carcass as % of body weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.28$, $p<0.0001$ Ratio of IGF-I/IGFBP-3 -, $\delta r^2 = 0.19$, $p<0.05$	

Table 3.22 Summary of independent covariates identified as predictors of tissue weights in the pregnant and nonpregnant guinea pig. A combination of eight independent variables the ratio of IGF-I, -II to IGFBP-1, -2, -3, -4 were included in the analysis.

Dependent variable	**Independent Covariates ratio of IGFstoIGFBPs**	
Tissue weight (g)	**Pregnant**	**Nonpregnant**
Uterus, g	Ratio of IGF-I/IGFBP-2 +, δ r^2 = 0.52, p<0.01 Ratio of IGF-II/IGFBP-2 -, δ r^2 = 0.13, p<0.05	Ratio of IGF-II/IGFBP-4 +, δ r^2 = 0.3, p<0.05
Retroperitoneal fat, g	Ratio of IGF-I/IGFBP-2 +, δ r^2 = 0.74, p<0.0001	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.54, p<0.005 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.24, p<0.005
Dorsal fat, g	Ratio of IGF-I/IGFBP-1 +, δ r^2 = 0.74, p<0.001 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.12, p<0.01	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.58, p<0.001 Ratio of IGF-I/IGFBP-1 -, δ r^2 = 0.17, p<0.02
Total gastrointestinal tract, g	Ratio of IGF-I/IGFBP-2 -, δ r^2 = 0.35, p<0.02	Ratio of IGF-II/IGFBP-1 -, δ r^2 = 0.53, p<0.002
Spleen, g	Ratio of IGF-I/IGFBP-1 +, δ r^2 = 0.59, p<0.001	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.65, p<0.0001 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.13, p<0.02
Thymus, g	Ratio of IGF-I/IGFBP-1 +, δ r^2 = 0.73, p<0.0001 Ratio of IGF-II/IGFBP-3 -, δ r^2 = 0.08, p<0.05	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.63, p<0.001 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.12, p<0.05
Soleus, g	Ratio of IGF-I/IGFBP-1 +, δ r^2 = 0.79, p<0.0001 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.06, p<0.05	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.8, p<0.0001 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.09, p<0.01
Liver, g	Ratio of IGF-I/IGFBP-1 +, δ r^2 = 0.67, p<0.0001 Ratio of IGF-II/IGFBP-3 -, δ r^2 = 0.08, p=0.05	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.65, p<0.001 Ratio of IGF-I/IGFBP-1 -, δ r^2 = 0.14, p<0.02

Table 3.23 Summary of independent covariates identified as predictors of body phenotype in *ad libitum* fed and underfed guinea pigs. A combination of eight independent variables the ratio of IGF-I, -II to IGFBP-1, -2, -3, -4 were included in the analysis.

Dependent variable	Independent Covariates ratio of IGFs/IGFBPs	
	***ad libitum* fed, pregnant and nonpregnant**	**Underfed, pregnant and nonpregnant**
Body weight	Ratio of IGF-I/IGFBP-3 +, $\delta\ r^2 = 0.48$, $p<0.001$ Ratio of IGF-II/IGFBP-3 -, $\delta\ r^2 = 0.24$, $p<0.02$	Ratio of IGF-II/IGFBP-1 -, $\delta\ r^2 = 0.35$, $p<0.005$ Ratio of IGF-I/IGFBP-3 +, $\delta\ r^2 = 0.16$, $p<0.05$
Net body weight		
Weight gain	Ratio of IGF-I/IGFBP-3 +, $\delta\ r^2 = 0.61$, $p<0.001$ Ratio of IGF-II/IGFBP-3 -, $\delta\ r^2 = 0.19$, $p<0.02$	Ratio of IGF-II/IGFBP-1 -, $\delta\ r^2 = 0.57$, $p<0.001$
Net weight gain		Ratio of IGF-II/IGFBP-3 -, $\delta\ r^2 = 0.31$, $p<0.02$
Carcass weight		
Carcass as % of body weight		

Table 3.24 Summary of independent covariates identified as predictors of tissue weights in *ad libitum* fed and underfed guinea pigs. A combination of eight independent variables the ratio of IGF-I, -II to IGFBP-1, -2, -3, -4 were included in the analysis.

Dependent variable	**Independent Covariates ratio of IGFs/IGFBPs**	
Tissue weight (g)	***ad libitum* fed ,pregnant and nonpregnant**	**Underfed, pregnant and nonpregnant**
Uterus, g	Ratio of IGF-I/IGFBP-3 +, δ r^2 = 0.67, p<0.0001 Ratio of IGF-II/IGFBP-3 -, δ r^2 = 0.22, p<0.001	Ratio of IGF-II/IGFBP-2 -, δ r^2 = 0.28, p<0.002 Ratio of IGF-II/IGFBP-1 -, δ r^2 = 0.26, p<0.02 Ratio of IGF-I/IGFBP-3 +, δ r^2 = 0.19, p<0.001
Retroperitoneal fat, g		
Dorsal fat, g		Ratio of IGF-II/IGFBP-1 +, δ r^2 = 0.22, p<0.05
Total gastrointestinal tract, g		Ratio of IGF-II/IGFBP-1 -, δ r^2 = 0.4, p<0.01
Spleen, g		
Thymus, g	Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.66, p<0.0001 Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.17, p<0.002 Ratio of IGF-I/IGFBP-4 +, δ r2 = 0.08, p<0.02	Ratio of IGF-II/IGFBP-2 +, δ r^2 = 0.34, p<0.02
Soleus, g	Ratio of IGF-I/IGFBP-1 +, δ r^2 = 0.7, p<0.0001 Ratio of IGF-I/IGFBP-2 +, δ r^2 = 0.1, p<0.01 Ratio of IGF-I/IGFBP-3 -, δ r^2 = 0.09, p<0.02	Ratio of IGF-II/IGFBP-1 +, δ r^2 = 0.33, p<0.02
Liver, g		

	IGF-I mRNA	Plasma IGF-I ng/ml	Plasma IGF-II ng/ml	IGFBP-3	IGFBP-2
Fetuses per dam	0.39, *NS*	0.61, $p = 0.01$	0.47, *NS*	0.31, *NS*	-0.16, *NS*
Average fetal weight	0.58, $p = 0.022$	0.77, $p < 0.001$	0.73, $p < 0.01$	0.77, $p < 0.001$	-0.72, $p < 0.01$
Average placental weight	0.41, *NS*	0.59, $p < 0.05$	0.54, $p < 0.05$	0.63, $p < 0.01$	-0.74, $p < 0.001$
Average fetal /placental weight	0.54, $p < 0.05$	0.69, $p < 0.01$	0.69, $p < 0.01$	0.6, $p < 0.05$	-0.37, *NS*
Total fetal weight	0.64, $p < 0.01$	0.84, $p < 0.0001$	0.7, $p < 0.01$	0.74, $p < 0.01$	-0.57, $p = 0.02$
Total fetal weight as % of body weight	0.56, $p < 0.05$	0.76, $p < 0.001$	0.64, $p < 0.01$	0.51, $p < 0.05$	-0.41, *NS*
Total fetal weight as % of carcass weight	0.54, $p < 0.05$	0.71, $p < 0.01$	0.58, $p < 0.02$	0.45, *NS*	-0.33, *NS*
Total placental weight	0.55, $p = 0.03$	0.79, $p < 0.001$	0.66, $p < 0.01$	0.64, $p < 0.01$	-0.54, $p = 0.03$
Total placental weight as % of body weight	0.38, *NS*	0.62, $p < 0.05$	0.49, $p < 0.05$	0.29, *NS*	-0.31, *NS*
Total placental weight as % of carcass weight	0.36, *NS*	0.57, $p < 0.05$	0.44, *NS*	0.24, *NS*	-0.22, *NS*

Table 3.25 Associations between hepatic IGF-I mRNA or maternal endocrine IGF axis and fetal and placental weights. Pearson correlation coefficients r are tabulated and p is significant at $p<0.05$.

Tissue weight (g)	IGF-I/IGFBP-3	IGF-I/IGFBP-1	IGF-I/IGFBP-2	IGF-I/IGFBP-4	IGF-II/IGFBP-1	IGF-II/IGFBP-2
Uterus, g		0.69, p<0.005	0.72, p<0.002	0.51, p<0.05	0.55, p<0.05	0.6, p<0.02
Uterus as % of body weight		0.57, p<0.02	0.62, p<0.02			0.5, p<0.05
Uterus as % of carcass weight	0.53, p<0.05	0.54, p<0.05	0.54, p<0.05			
Fetuses per dam	0.6, p<0.02					
Average fetal weight		0.74, p<0.002	0.77, p<0.001	0.53, p<0.05	0.63, p<0.01	0.73, p<0.005
Average placental weight		0.65, p<0.01	0.75, p<0.001		0.56, p<0.05	0.72, p<0.002
Average fetal /placental weight		0.53, p<0.05				
Total fetal weight		0.68, p<0.005	0.72, p<0.002	0.51, p<0.05	0.55, p<0.05	0.6, p<0.02
Total fetal weight as % of body weigh	0.51, p<0.05	0.57, p<0.02	0.62, p<0.02			0.51, p<0.05
Total fetal weight as % of carcass weight	0.53, p<0.05	0.53, p<0.05	0.54, p<0.05			
Total placental weight		0.66, p<0.01	0.72, p<0.002	0.5, p<0.05	0.54, p<0.05	0.61, p<0.02
Total placental weight as % of body weight	0.62, p<0.001		0.53, p<0.05			
Total placental weight as % of carcass weight	0.63, p<0.01					

Table 3.26 Associations between the ratio of plasma IGFs to plasma IGFBPs and maternal and fetal tissues. Uterine weight (weight of the uterus, fetus and placenta). Pearsons correlation coefficients r are tabulated and p is significant at $p<0.05$

Table 3.27 Identification of independent covariates as predictors of fetal and placental weights (dependent variables) and IGF-I endocrine variables (independent variables).

Dependent variable	Pregnant	Pregnant feed restricted only
Fetuses per dam	IGF-I +, δ $r^2 = 0.4$, $p<0.02$	IGF-I +, δ $r^2 = 0.63$, $p<0.005$ IGFBP-2 +, δ $r^2 = 0.19$, $p<0.05$ IGFBP-4 -, δ $r^2 = 0.1$, $p=0.057$
Total fetal weight, g	IGF-I +,δ,$r^2 = 0.72$, $p<0.001$	
Total fetal weight as % of body weight	IGF-I +, δ $r^2 = 0.58$, $p<0.001$	
Total fetal weight as % of carcass weight	IGF-I +, δ $r^2 = 0.51$, $p<0.002$	
Total placental weight, g	IGF-I +,δ $r^2 = 0.63$, $p<0.001$	IGF-II +, δ $r^2 = 0.5$, $p<0.05$
Total placental weight as % of body weight	IGF-I +, δ $r^2 = 0.38$, $p<0.02$	
Total placental weight as % of carcass weight	IGF-I +, δ $r^2 = 0.33$, $p<0.02$	
Average fetal weight, g	IGFBP-3, +, δ $r^2 = 0.59$, $p<0.02$ IGF-II +, δ $r^2 = 0.11$, $p=0.053$	
Average placental weight, g	IGFBP-2 -, δ $r^2 = 0.55$, $p<0.001$	
Average fetal /placental weight ratio	IGF-I +, δ $r^2 = 0.48$, $p<0.005$	

Table 3.28 Summary of predictors of fetal and placental tissue weights (dependent variable) using the ratios of plasma IGFs to plasma IGFBPs as independent variables.

Covariate Analysis

Dependent variable	Independent variable
Total uterus weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.52$, $p<0.01$ Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.13$, $p<0.05$
Total Fetal weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.52$, $p<0.01$ Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.12$, $p=0.05$
Total Placental weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.52$, $p<0.01$ Ratio of IGF-II/IGFBP-2 +, $\delta r^2 = 0.12$, $p=0.05$
Average Fetal weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.59$,$p<0.001$
Average Placental weight	Ratio of IGF-I/IGFBP-2 +, $\delta r^2 = 0.55$, $p<0.001$ Ratio of IGF-I/IGFBP-3 +, $\delta r^2 = 0.14$, $p<0.05$
Average Fetal/Placental weight	Ratio of IGF-I/IGFBP-1 +, $\delta r^2 = 0.29$, $p<0.05$

Table 3.29 Identification of independent covariate as predictors of fetal and placental weight in *ad libitum* fed and feed restricted pregnant guinea pigs. A combination of eight independent variables using the ratio of plasma IGFs to plasma IGFBPs as independent variables.

	Pregnant	
Dependent variable	***ad libitum* fed** n = 8	**feed restricted** n = 8
Fetuses per dam	Ratio of IGF-II/IGFBP-4 -, $\delta\ r^2 = 0.64$, $p<0.02$	Ratio of IGF-I/IGFBP-1 +, $\delta\ r^2 = 0.69$, $p<0.01$
Total fetal weight, g	Ratio of IGF-II/IGFBP-4 -, $\delta\ r^2 = 0.58$, $p<0.05$	Ratio of IGF-I/IGFBP-1 +, $\delta\ r^2 = 0.43$, $p=0.07$
Total placental weight, g		Ratio of IGF-I/IGFBP-1 +, $\delta\ r^2 = 0.66$, $p<0.02$
Average fetal weight, g		
Average placental weight, g		
Average fetal /placental weight ratio		

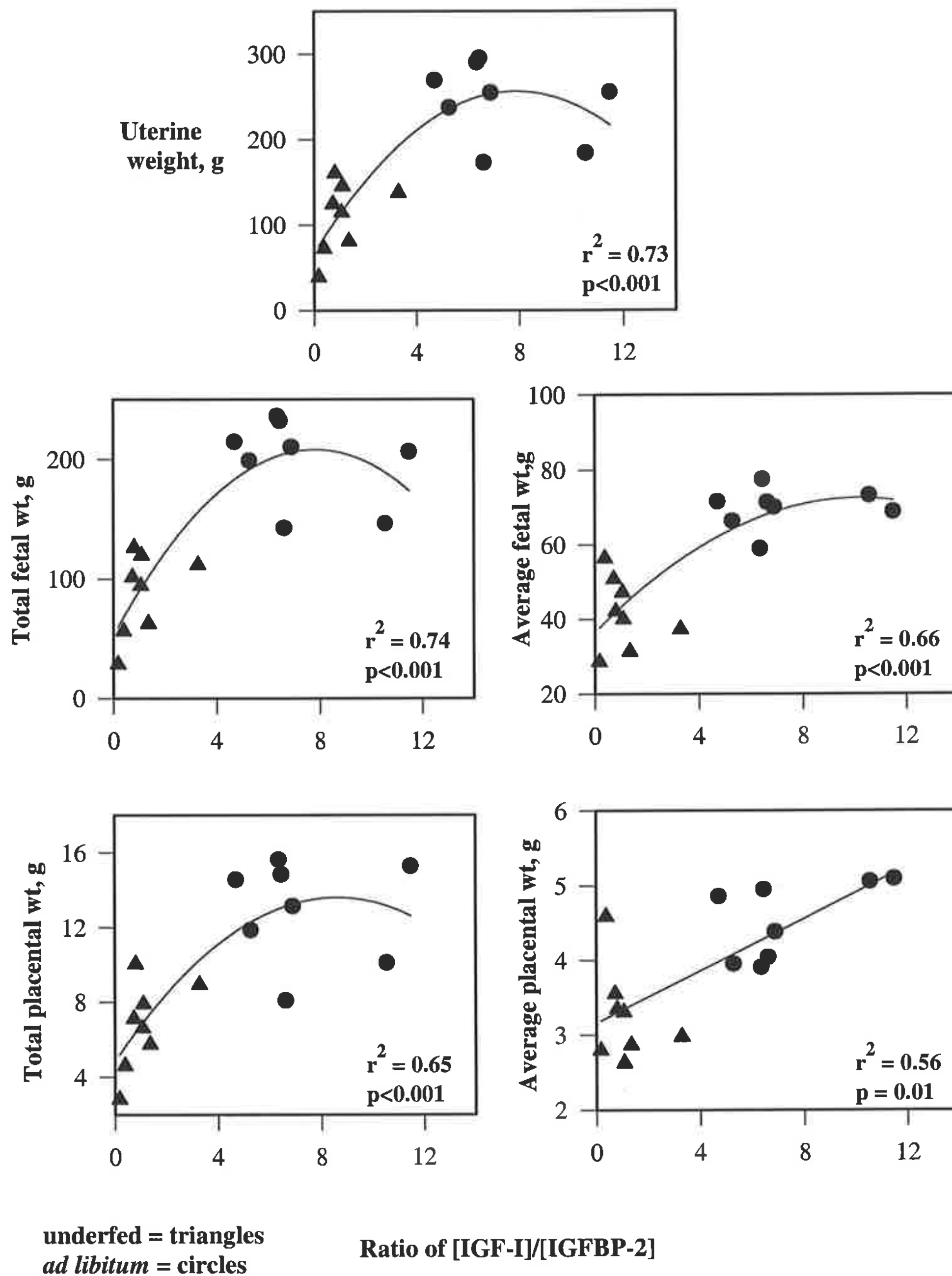


Figure 3.13 Correlations between uterine components and the ratio of the concentrations of IGF-I and IGFBP-2 in plasma from pregnant guinea pigs.

3.4 DISCUSSION

3.4.1 Endocrine IGF axis in pregnancy

The concentration of plasma IGF-I in guinea pigs in late pregnancy is relatively high (Sohlstrom et al., 1998), as seen in pregnant humans (Dwyer and Stickland, 1992;Gargosky et al., 1990a). This contrasts with mice, rats and rabbits where plasma IGF-I levels are high in mid-pregnancy but are very low late in pregnancy (Fielder et al., 1990;Gargosky et al., 1990b;Nason et al., 1996). The mechanism of increased plasma IGF-I in human pregnancy has yet to be defined. In rats, pregnancy-induced changes in plasma IGF-I were not related to hepatic abundance of IGF-I mRNA (Davenport et al., 1990;Monaco and Donovan, 1997;Travers et al., 1993).

3.4.2 Hepatic expression of IGF-I mRNA

The present study demonstrates that hepatic expression of IGF-I mRNA is increased by late pregnancy in guinea pigs. This effect was eliminated by feed restriction. As previously reported, plasma IGF-I follows a similar pattern (Sohlstrom et al., 1998). Plasma IGF-I was positively correlated ($r^2 = 0.68$) with the relative abundance of IGF-I mRNA in liver. In other words, 68% of the variation in plasma IGF-I can be accounted for by variation in hepatic abundance of IGF-I mRNA. This strongly indicates that synthesis by the liver is the major source of endocrine IGF-I in guinea pigs in late pregnancy. The novel Cre/loxP system used to ablate the IGF-I gene in the murine liver resulted in a 75% reduction of plasma IGF-I, confirming the liver as the primary source of endocrine IGF-I in nonpregnant mice (Yakar et al., 1999).

3.4.3 Mechanisms of hepatic IGF-I mRNA regulation in pregnancy

Placental growth factors

Regulation of expression of hepatic IGF-I mRNA in pregnancy is not completely understood. Placental hormones have been suggested as potential candidates. Several studies have suggested placental growth hormone-like peptides, such as placental lactogen and placental growth hormone may act through hepatic growth hormone receptors (GHR) stimulating the expression of hepatic IGF-I thereby increasing endocrine IGF-I. Other candidates include estrogen, progesterone and cortisol.

Regulation of hepatic IGF-I in pregnancy may follow the original somatomedin hypothesis where GH or GH-like polypeptides of placental origin stimulate hepatic IGF-I production via interaction with the transmembrane GHR present on the surface of liver cells (Le Roith, 2001). Because of the high degree of primary sequence homology between GH, PL and placental GH it is probable that one of these placental GH-like peptides may interact with the hepatic GHR. This does not exclude the possibility that these placental peptides act through distinct alternate receptors.

Placental Lactogen

Mice and rat placentae secrete two forms of placental lactogen (PL). PL-1 is secreted early in pregnancy and PL-2 in the second half of pregnancy in mice (Faria et al., 1990). PL-1 and PL-2 mRNA are expressed and the proteins are produced by trophoblast giant cells of mouse placenta (Faria et al., 1990). Synctiotrophoblast cells of the human placenta synthesise a single PL (Handwerger, 1991). Maternal plasma concentrations of placental lactogen parallel the increase in maternal concentrations of plasma IGF-I and they are weakly correlated with each other from weeks 30 to 31 and weeks 32 to 33 in human pregnancy (Caufriez et al.,

1993). Placental lactogen has been proposed as a regulator of hepatic IGF-I mRNA through either the growth hormone receptor or an unidentified placental lactogen receptor. Ovine placental lactogen and ovine growth hormone were demonstrated to bind a common receptor in microsomal membranes isolated from pregnant sheep liver (Breier et al., 1994). Ovine placental lactogen had a 10-fold greater affinity than ovine GH for this receptor. Breier *et al* (1994) suggested that oPL binds to a single subunit of the GHR initiating signal transduction whereas oGH requires dimerization of two GHR subunits to initiate receptor signalling. Thus the greater potency of oPL for the receptors isolated from pregnant sheep liver was possibly achieved by binding one molecule of oPL to one subunit of GHR whereas one molecule of oGH requires two subunits of GHR (Breier et al., 1994).

Placental growth hormone

Human placental growth hormone is expressed by syncytiotrophoblasts of the placenta and secreted into the maternal circulation from week 15 to 20, progressively increasing to term (Alsat et al., 1998). Growth hormone receptors are present in the placenta suggesting placental growth hormone may have an autocrine role (Alsat et al., 1998). Maternal plasma concentrations of IGF-I are positively correlated with those of placental growth hormone (Caufriez et al., 1993;Caufriez et al., 1994;Evain-Brion, 1999;McIntyre et al., 2000). Mothers of IUGR babies have lower plasma concentrations of both IGF-I and placental growth hormone (Mirlesse et al., 1993). Thus placental growth hormone and/or placental lactogen may use a mechanism similar to that suggested as the original somatomedin hypothesis of pituitary growth hormone action on hepatic IGF-I production.

Methylation of placental hormones

A mechanism that potentially may regulate the expression and concentration of placental growth hormone like peptides is DNA methylation. Methylation of the rat placental lactogen-1 gene has recently been suggested as a mechanism regulating tissue specific expression of

placental lactogen (Cho et al., 2001). As the PL and PL-like genes are co-localized in a cluster, DNA methylation may affect gene expression of these PL-like factors. An inverse relationship between DNA methylation and tissue expression has been shown for rat prolactin and growth hormone genes (Ngo et al., 1996). The degree of DNA methylation can be influenced by the nutritional status of the animal (Hass et al., 1993). Factors such as folate and methionine are important precursors of S-adenosylmethionine the major methyl donor in the body. Thus, caloric deprivation may affect DNA methylation as seen in pancreatic acinar cells from undernourished rats where genomic methylation of ras DNA was increased (Slattery et al., 1997). Therefore expression of PL-like genes can be affected by DNA methylation, which is potentially dependent on dietary status.

3.4.4 Nutritional regulation of hepatic IGF-I mRNA

Undernutrition in pregnant rats and guinea pigs decreases plasma concentrations of plasma IGF-I (Muaku et al., 1995a;Muaku et al., 1995b;Sohlstrom et al., 1998). This study shows for the first time that hepatic expression of IGF-I mRNA is reduced by feed restriction in pregnant guinea pigs. Mechanisms regulating hepatic production of IGF-I in food restricted animals appear to be independent of growth hormone secretion, but are dependent on energy intake and availability of essential amino acids (Zhang et al., 1998).

Fasting of pregnant ewes for 72 h was shown to decrease placental lactogen binding to maternal hepatic microsomal membranes by 70% (Freemark et al., 1992). Infusion of glucose during this time failed to restore placental lactogen binding. Other studies have shown that refeeding restored placental lactogen binding, suggesting that nutrients other than glucose, such as amino acids and fatty acids, regulate abundance of placental lactogen receptors.

Growth hormone resistance produced by undernutrition has been shown to prevent GH dependent IGF-I stimulation in undernourished nonpregnant steers (Breier, 1999). However, the numbers of growth hormone receptors were minimally decreased by nutritional constraint suggesting the lack of IGF-I response to GH administration may be due to postreceptor defects (Thissen et al., 1994). Reduced total energy intake and reduced protein intake correlates with decreased hepatic expression of IGF-I mRNA. Protein restriction decreases the availability of essential amino acids, which are required for expression of hepatic IGF-I mRNA. Both fasting and protein restriction have been demonstrated to reduce transcription and translation of hepatic IGF-I mRNA (section 1.5.2) (Thissen et al., 1991;Zhang et al., 1998;Zhang et al., 1997).

3.4.5 Pregnancy changes the relationship between plasma concentrations of IGF-I and hepatic abundance of IGF-I mRNA

Alternate forms of hepatic IGF-I mRNA transcripts

The second order nature of the relationship between hepatic abundance of IGF-I mRNA and circulating protein may be a result of altered translation of IGF-I or accumulation of intermediate RNA transcripts of larger size that are not translated (Hepler et al., 1990). Protein and caloric restriction have been shown to alter the molecular profile of rat IGF-I transcripts, as seen on Northern blot analysis (Zhang et al., 1998).

Alternatively placental lactogen and placental growth hormone may differentially regulate hepatic expression of different forms of IGF-I transcript class 1 and class 2. Class 1 and 2 transcripts are regulated by pituitary GH and nutrition, although class 2 transcripts are more sensitive to growth hormone and higher dietary intake of protein and energy intake (Pell et al., 1993;Weller et al., 1993). GH and higher nutrition act synergistically to increase the expression of IGF-I class 2 transcripts in ovine liver (Pell et al., 1993). This synergistic effect

appears similar to that observed between pregnancy and higher nutrition in guinea pig liver on the relative abundance of IGF-I mRNA. Circulating IGF-I protein was more strongly associated with class 2 transcripts than class 1 in sheep, suggesting this form may be responsible for changes in circulating IGF-I. Pell *et al* (1993) proposed that the relative amount of class 2 to class 1 transcripts act as a switch to determine the endocrine secretion of IGF-I into blood. In pregnant *ad libitum* fed guinea pigs, the relative abundance of hepatic IGF-I mRNA may be equivalent to class 1 plus class 2 transcripts (6 fold increase), while plasma IGF-I (3 fold increase) may correlate with only class 2 transcripts. The large discrepancy between the change in plasma IGF-I concentration and hepatic IGF-I mRNA abundance in *ad libitum* fed guinea pigs caused by pregnancy may therefore be explained by pregnancy specific factors increasing IGF-I class 1 transcripts, which do not lead to circulating IGF-I protein, as well as class 2 transcripts which are translated.

In rodents the IGF-I gene has two leader exons (exon 1 and 2) producing two kinds of mRNAs, class 1 and class 2, although a third mRNA is present designated class 1 del (mRNA lacking a segment of exon 1) (Holthuizen, 1991). Mouse Ob1771 preadipocyte cells cultured with growth hormone differentiate to adipocytes and express class I IGF-I mRNA and class 1 del IGF-I mRNA. These preadipocytes when cultured with GH and IGF-I stimulate both classes of transcripts with class 1 IGF-I mRNA the major form. The class 1 IGF-I mRNA species was translated more efficiently than the class 1 del IGF-I mRNA. Kamai *et al* (1996) hypothesised that GH stimulates the initial synthesis of a less efficiently translated class 1 del IGF-I mRNA, which subsequently stimulates class I IGF-I mRNA. Therefore in pregnancy placental lactogen or placental growth hormone may initially stimulate the expression of the less stable IGF-I class 1 del mRNA, which does not lead to circulating protein but may also regulate the expression of IGF-I transcripts that contribute to the concentration of IGF-I in blood.

3.4.6 Pregnancy increases the clearance rate of endocrine IGF-I from blood

As discussed above, the relationship between hepatic abundance of IGF-I mRNA and plasma IGF-I concentrations, while very strong, is not linear. In *ad libitum* fed guinea pigs, pregnancy increased plasma IGF-I concentration about 3-fold whereas hepatic abundance of IGF-I mRNA was increased about 6-fold. As an alternative to the mechanism described above this discrepancy could be due to proteolytic conversion of a proportion of IGF-I protein to the des-(1-3) IGF-I variant which is indistinguishable from IGF-I in the RIA and is cleared from blood more rapidly. Des-(1-3) IGF-I has reduced affinity for IGFBPs and is cleared more rapidly from the circulation than either IGF-I or IGF-II in rats (Ballard et al., 1991;Gillespie et al., 1990). The formation of this variant is thought to be a posttranslational modification of the mature IGF-I. Several studies support this view including the identification of a trypsin-like acid protease capable of cleaving des-(1-3) IGF-I from the mature IGF-I (Yamamoto and Murphy, 1994). Hypophysectomized rats have increased activity of a protease that can convert IGF-I to the des-(1-3) IGF-I in many tissues with the highest being in the liver. Treatment with growth hormone reversed this (Yamamoto and Murphy, 1995). In human pregnancy pituitary growth hormone levels in blood decline while a continuous secretion of placental growth hormone increases. Reduction of pituitary GH in pregnancy may act to increase protease activity in the liver thus increasing production of des-(1-3) IGF-I. The activity of the acid protease responsible for generating des-(1-3) IGF-I from IGF-I has been demonstrated to be inhibited by a growth hormone dependent serine protease inhibitor Spi 2.1 (Maake et al., 1997).

If hepatic synthesis and secretion of IGF-I is directly linearly related to hepatic abundance of IGF-I mRNA in guinea pigs then a change in the ratio of plasma IGF-I to IGF-I mRNA indicates that the rate of clearance of IGF-I from the blood of *ad libitum* fed animals was

doubled by pregnancy. Pregnancy has been reported to increase clearance of IGF-I from the circulation in rats (Bastian et al., 1993;Davenport et al., 1990).

A synthetic IGF-I analogue LR^3IGF-I, known to have very poor affinity for IGFBPs, is more rapidly cleared than IGF-I in nonpregnant and pregnant rats. This demonstrates that association with IGFBPs extends the half-life of circulating IGFs. Radiolabelled IGF-I and LR^3IGF-I were found localised in different tissues after intravenous injection into pregnant and nonpregnant rats, suggesting transportation by IGFBPs plays a significant role in targeting IGF-I to tissues (Bastian et al., 1993). Uptake of radiolabelled IGF-I was higher than that of LR^3IGF-I in the placenta, fetus and fetal plasma. Thus the ability of IGF-I to bind IGFBPs in pregnancy appears important in the uptake or action at the maternal-fetal interface.

Insulin-like growth factor binding proteins play a very significant role in the delivery, availability and activity of IGFs. In guinea pigs, as in other mammals, plasma IGFBPs were altered by pregnancy and nutrition. However, unlike most mammals, pregnancy had no discernible effect on plasma IGFBP-3 in guinea pigs. Also, there was no evidence of IGFBP-3 proteolytic activity in guinea pig plasma. This contrasts with human and rat where pregnancy increases plasma IGFBP-3 proteolysis (Binoux et al., 1993;Davenport et al., 1992;Gargosky et al., 1992). In feed restricted guinea pigs, however, pregnancy increased concentrations of IGFBP-1 and IGFBP-2 and decreased those of IGFBP-3 in plasma.

3.4.7 Potential actions of endocrine IGF-I in pregnancy

The relative abundance of IGF-I mRNA in liver and the concentration of IGF-I in plasma of pregnant guinea pigs were both positively correlated with maternal body weight, net body weight, weight gain, net weight gain and uterine weight, the latter both as a fraction of body weight and carcass weight, as well as weights of retroperitoneal fat, dorsal fat, spleen and

thymus. This suggests that IGF-I synthesised and secreted by the liver may have endocrine actions on maternal tissues during pregnancy. This is supported by the observation that infusion of IGF-I (350 μg/day) into late pregnant rats increased maternal weight gain, but had no effect on placental or fetal weight (Gargosky et al., 1991). More recently, however, administration of IGF-I (565 μg/day) to *ad libitum* fed guinea pigs from day 20 to 37 of pregnancy failed to increase maternal body weight, but increased fetal and placental weight (Sohlstrom et al., 2001).

In nonpregnant animals the relative abundance of hepatic IGF-I mRNA was not significantly related to the weight of any tissue except liver. This suggests that IGF-I may have autocrine or paracrine actions on the liver and somatic growth of nonpregnant guinea pigs is not dependent on endocrine actions of hepatic IGF-I. Somatic growth of nonpregnant guinea pigs is reported to be independent of pituitary GH (Keightley and Fuller, 1996). Passive immunoneutralization of endocrine IGF-I in male guinea pigs had no effect on growth rate or weight of tissues (Kerr et al., 1990).

In the nonpregnant guinea pig, plasma IGF-I positively correlated with the weights of retroperitoneal fat, dorsal fat, spleen, thymus and soleus muscle. However plasma IGF-II was a better correlate with almost all tissue and organ weights in nonpregnant guinea pigs, suggesting IGF-II is normally a more important endocrine growth factor than IGF-I in this species.

In this present study covariate analysis was performed with six independent variables, the plasma concentrations of IGF-I, IGF-II and IGFBP-1, -2, -3 and –4, to determine if any of these factors were independently related to tissue and organ weights. In pregnant guinea pigs, IGF-II (n = 10, where n is the number of variables that correlated with an independent IGF endocrine factor) or IGFBP-3 (n = 2) concentrations in plasma were the main independent

covariates of organ and tissue weights. This contrasts with the situation in pregnancy, where the plasma concentrations of IGF-I (n = 12) or IGFBP-3 (n = 8) were the most frequently observed independent covariates of tissue and organ weights.

In pregnant animals, the concentration of either IGF-I or IGFBP-3 was found to be a better independent covariate of all organ and tissue weights than the concentration of IGF-II or any other IGFBP in blood (Table 3.18, 3.19, 3.27). The only exception is that the concentration of IGFBP-2 in plasma is the strongest correlate of fractional carcass weight in pregnancy. Correlations between tissue weights and the concentrations of IGFBP-1, -2 and -4 in pregnant guinea pigs were generally weak and negative except for average placental weight where the concentration of IGFBP-2 is a major independent covariate (Table 3.27).

Plasma concentration of IGFBP-3 was a strong and positive independent predictor of spleen and thymus weight in pregnant guinea pigs, suggesting IGFs associated with IGFBP-3 may be important in regulating the immune system. IGF-I stimulates lymphocyte maturation through the type 1 IGF receptor (Kooijman et al., 1995). IGF-I has also been demonstrated to alter the ratio of CD4 to CD8 lymphocytes in the thymus, which may be important in the development of immune tolerance in pregnancy. In pregnant mice the thymus shrinks and rearrangement of the internal structure occurs which may be involved with the development of maternal tolerance of paternal and fetal antigens (Clark, 1997). Administration of IGF-I to the fetal rhesus monkey increased thymus, spleen, kidney and small intestine weights as well as the number of red blood cells, lymphocytes and neutrophils in the third trimester. The ratio of CD4 to CD8 lymphocytes was also increased by IGF-I in fetal lymph nodes (Tarantal et al., 1997). Total gastrointestinal weight in pregnant guinea pigs was negatively related to plasma IGF-I, suggesting the anabolic action of endocrine IGF-I was inhibited possibly by circulating IGFBPs in plasma. Findings of this current study imply IGF-I and/or IGFBP-3, either in combination or independently, may play an important role in the immune system.

The concentration of plasma IGFBP-3 was a major independent covariate of the weights of retroperitoneal and dorsal fat in pregnant animals. In nonpregnant guinea pigs IGFBP-3 was a major independent predictor of retroperitoneal fat and soleus muscle weight. These strong positive correlations suggest IGFBP-3 may deliver IGFs to these tissues. However some IGFBP-3 actions are known to be independent of either IGF-I or IGF-II (Baxter, 2000;Wetterau et al., 1999).

3.4.8 Nutritional regulation of endocrine IGF axis in pregnant and nonpregnant guinea pigs

In *ad libitum* fed animals the plasma concentration of IGF-I was a major independent covariate of total body weight, weight gain and uterine weight. Correlation between thymus weight and the concentration of IGF-I in *ad libitum* fed animals was strong and negative with IGF-II, IGFBP-3 and IGFBP-4 as minor independent covariates. Soleus muscle weight correlated positively with the concentration of IGF-I and negatively with the concentration of IGFBP-1 in *ad libitum* fed guinea pigs. The positive association of soleus muscle weight with IGF-I and negative association with IGFBP-1 suggest they may interact to maintain homeostatic balance between synthesis and degradation of protein in muscle (Oddy and Owens, 1996).

In feed restricted animals the plasma concentration of IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 were found to be weak independent positive covariates of tissue and organ weights, except for thymus weight where the relationship was negative (Table 3.18, 3.20). The concentration of IGF-II and IGFBP-1 were equal positive independent covariates of net body weight. The concentration of IGF-I was a weak independent covariate of liver weight in

underfed animals. These associations may indicate that all IGFBPs may act to increase the bioavailability of limited circulating IGF-I and II to target tissues promoting their growth.

In nutritionally restricted dams the concentrations of IGF-I and IGF-II in blood were found to be major independent covariates of number of fetuses per dam and total placental weight respectively (Table 3.27).

Nutritional restriction decreases plasma IGF-I, which may limit the number of fetuses supported in pregnancy. IGF-II has been suggested to have an important role in determining placental size by promoting trophoblast invasion into maternal decidua (Hamilton et al., 1998).

3.4.9 Potential function of circulating complexes between IGFs and IGFBPs

In pregnant guinea pigs undernutrition increased plasma concentrations of IGFBP-1 and -2 and decreased those of IGFBP-3, IGF-I and IGF-II, suggesting that changes in the concentrations of IGFBPs and IGFs may contribute significantly to both fetal and placental growth (Sohlstrom et al., 1998) because the bioavailability of IGFs is dependent on plasma concentrations of IGFBPs and IGFs.

The ratios of the concentrations of IGF-I and IGF-II to that of IGFBP-1, -2, -3 and -4 in plasma are indicators of potential amounts of circulating complexes in blood. The theoretical interpretation of the relationships between tissue weights and the ratios of the concentrations of IGFs to IGFBPs are as depicted in Figure 3.14. A relatively high ratio of the concentrations of IGF-I or IGF-II to those of a particular binding protein, indicate that a relatively large amount of the IGF is not complexed to that particular binding protein. A positive relationship between tissue weight and the ratio of plasma IGF to IGFBP concentrations therefore means

that particular binding protein inhibits growth promoting endocrine actions of IGF (-1 or –II) on that particular tissue.

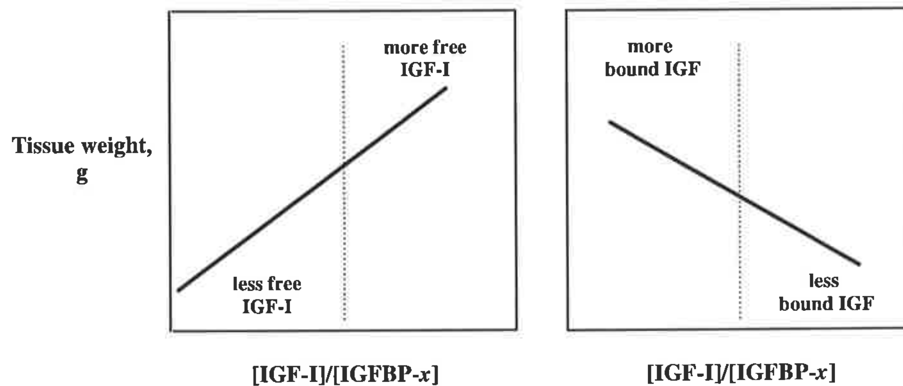


Figure 3.14 Theoretical model showing the two possible relationships between the ratio of the concentration of IGFs to IGFBPs and tissue weights. A positive relationship indicates IGFBP restricts IGF actions on tissue growth. While a negative relationship indicates tissue growth is greatest when IGF is complexed with IGF binding proteins.

A negative relationship between weight of a tissue and the ratio of the concentrations of IGF (-I or –II) to those of a particular binding protein means that tissue growth is greatest when there is a high percentage of these particular IGF/IGFBP complexes. This suggests that complexes between IGF (-I or –II) and this particular binding protein promote growth of this particular tissue.

All potential measures of circulating complexes of IGF-I or IGF-II to IGFBP-1, -2, -3 and -4 were investigated by forward stepwise regression. In pregnancy almost all the significant correlations between tissue weights and ratio of concentrations of IGFs to IGFBPs in plasma involve either IGFBP-1 (n = 8) or IGFBP-2 (n=11) as major independent covariates. In nonpregnant animals, however, all but two correlations between tissue weights and these

measures of potential IGF to IGFBP complexes involve the ratio of concentration of IGF-II to IGFBP-2 (n = 11) as a major covariate.

Fifty to eighty percent of the variation in weights of tissues and organs in nonpregnant guinea pigs was determined independently by the ratio of the concentration of [IGF-II] to [IGFBP-2] in plasma. IGF-II was a more dominant factor than IGF-I in covariate analyses of IGF endocrine determinants of weights of tissues and organs in nonpregnant animals suggesting that IGF-II may have a greater endocrine role than IGF-I in nonpregnant guinea pigs.

Treatment of normal nonpregnant rats with IGF-II (650 μg/day) increased gut weight, kidneys, spleen, stomach and small intestine (Conlon et al., 1995a). However in guinea pigs administration of IGF-II (360 μg/day) over 7 days failed to increase any tissue weights, although plasma IGF-II was significantly increased (Conlon et al., 1995b). In contrast a*d libitum* fed pregnant guinea pigs treated with IGF-II (565 μg/day) had higher interscapular fat weight as a % of body weight as well as greater fetal and placental weights (Sohlstrom et al., 2001). Undernourished pregnant guinea pigs similarly treated with IGF-II had higher spleen weight only. In general administration of IGF-II to either pregnant or nonpregnant guinea pigs had little effect on adult tissue growth, although in pregnancy growth of both the placenta and fetus was observed. Simultaneous infusion of IGF-I (15 μg/kg.hr) and IGF-II (50 μg/kg.hr) into 4 month old lambs prevented the decrease in net protein catabolism seen with IGF-I (15 μg/kg.hr) only (Koea et al., 1992). It has been proposed that IGF-II may modulate IGF-I anabolic effects either by inhibition at the receptor site or by downregulation of the type I IGF receptor (Rosenthal et al., 1991). Also, unlike endogenous endocrine IGF production, administration of IGF-I (or IGF-II) reduces circulating levels of insulin and IGF-II (or IGF-I). Furthermore, IGF administration alters amounts of IGFBPs in blood in ways that are different from those produced by environmental regulation of endogenous production of IGFs.

The ratios of the concentrations of IGF-I to those of either IGFBP-1 ([IGF-I]/[IGFBP-1]) or -2 are better independent correlates than the ratios of the concentrations of IGF-II to those of any IGFBP for all tissue and organ weights measured in pregnancy (Table 3.21, 3.22, 3.28). This suggests that in general, IGF-I promotes growth of maternal organs in pregnancy and that IGFBP-1 and -2 inhibit this action. The only exception is that the ratio of plasma IGF-II to IGFBP-3 concentrations was the strongest correlate of net maternal weight gain during pregnancy. Net weight gain during pregnancy was negatively correlated with [IGF-II]/[IGFBP-3]. The [IGF-II]/[IGFBP-1] was a minor independent covariate of net weight gain during pregnancy (Table 3.21).

The negative relationship between net weight gain and the ratio of plasma [IGF-II] to [IGFBP-3] raise the possibility that IGF-II actions on maternal weight gain in pregnancy are dependent on formation of complexes with IGFBP-3. The positive association between net weight gain and the ratio of the [IGF-II] to [IGFBP-1] suggests IGFBP-1 inhibits these anabolic actions of IGF-II.

The above correlations between the ratio of [IGF-I] to either [IGFBP-1], [-2] and tissue weights in pregnancy are all positive except for the ratio of the [IGF-I] to [IGFBP-2] and weight of total gastrointestinal tract. This negative relationship between the ratio of the [IGF-I] to [IGFBP-2] and total weight of gastrointestinal tract may suggest that IGFBP-2 may preferentially deliver IGF-I to this organ possibly regulating the gut immune system to protect the mother from infection across the gut (Clark, 1997).

The ratio of [IGF-I] to [IGFBP-2] in plasma was an independent positive covariate of pregnant body weight, total weight gain during pregnancy, the weights of retroperitoneal fat, uterus, average and total fetal weight and average and total placental weight. The ratio of [IGF-I] to [IGFBP-1] was a positive correlate of pregnant net body weight at the end of

pregnancy and the weight of dorsal fat, spleen, soleus muscle, thymus and liver, as well as the average fetal to placental weight ratio (Table 3.21, 3.22, 3.28). This suggests endocrine IGF-I promotes growth of these tissues in pregnancy and that IGFBP-2 and IGFBP-1 inhibit these actions.

For example, dorsal fat weight in pregnancy was positively correlated with the ratio of [IGF-I] to [IGFBP-1]. This may indicate that the lipolytic effects of IGF-I are inhibited by IGFBP-1. Different fat depots are metabolically different and are differentially regulated (Wajchenberg, 2000). Visceral fat has higher lipolytic activity than subcutaneous fat and thus mobilises fatty acids more quickly (Wajchenberg, 2000). Visceral fat is highly sensitive to catecholamines and poorly sensitive to insulin's antilipolytic effect, whereas in subcutaneous fat the opposite is observed. There are more β-adrenoreceptors and fewer insulin receptors in visceral fat than in subcutaneous fat. High concentrations of IGFs can be antilipolytic when acting via the insulin receptor or lipolytic at low concentrations acting via the type I IGF receptor (Zapf, 1999). Undernutrition decreased the size of retroperitoneal and dorsal fat depots but pregnancy attenuated this effect in dorsal fat, suggesting that pregnancy may change the metabolic regulation of specific fat depots.

In pregnancy a strong positive association between soleus muscle weight and the ratio of the [IGF-I] to [IGFBP-1] in plasma was found, but this was not observed in nonpregnant animals. Thus, in pregnancy, the ratio of concentration of IGF-I to IGFBP-1 in plasma may potentially regulate muscle protein metabolism. IGFBP-1 inhibits IGF-I stimulated protein synthesis and glucose uptake in human skeletal muscle cells (Frost and Lang, 1999). IGFBP-1 also acts independently of IGFs by binding to β_1 integrin in human skeletal muscle cells inhibiting protein degradation (Frost and Lang, 1999). This mechanism may be important in partially controlling partitioning of nutrients between maternal tissues and the conceptus.

Correlations between maternal tissue weights and ratios of [IGF-I] or [IGF-II] to [IGFBP-3] in pregnancy were much less frequently observed than those involving [IGFBP-1] or [IGFBP-2]. They are also generally weak and negative.

Analysis of the dependence of fetal and placental tissue weights on the ratio of concentrations of IGF to IGFBP identified the ratio of [IGF-I] to [IGFBP-2] as a major independent covariate and the ratio of [IGF-II] to [IGFBP-2] as a minor independent covariate (Table 3.28). These relationships suggest that the ratio of the concentrations of IGF-I to IGFBP-2 in maternal plasma may have an important role in fetal and placental growth. The exceptions to this were average fetal to placental weight ratio where [IGF-I] to [IGFBP-1] was a minor independent covariate and average placental weight where the [IGF-I] to [IGFBP-3] was a minor independent covariate. The strong positive dependence of fetal and placental weights on the ratio of [IGF-I] to [IGFBP-2] in maternal plasma suggests the pregnant uterus, fetus and placenta are target tissues for IGF-I, while IGFBP-2 inhibits this action. The ratio of [IGF-I] to [IGFBP-1] was a major independent covariate of number of fetuses per dam and total fetal and placental weight in feed restricted animals. None of these correlations contained plasma IGF-II suggesting endocrine IGF-II does not contribute to fetal and placental growth late in gestation.

In *ad libitum* fed pregnant animals the ratio of [IGF-II] to [IGFBP-4] in plasma was a major independent covariate of number of fetuses per dam and total fetal weight. The ratio of [IGF-I] to [IGFBP-3] in *ad libitum* fed pregnant and nonpregnant animals is a major independent covariate of body weight, weight gain, uterine weight, thymus weight while [IGF-I] to [IGFBP-1] was a major independent covariate of soleus muscle weight (Table 3.23, 3.24).

Administration of an equimolar mixture of IGF-I and IGFBP-3 (5 μg/g of body weight) is reported to be more effective in increasing muscle protein synthesis in chronically semi-

starved rats than IGF-I alone (Svanberg et al., 2000). Improved bioavailability of IGF-I was also claimed when a combination of IGF-I and IGFBP-3 was administered to hypophysectomized rats (Adams et al., 1995;Zapf et al., 1995). Affinity between IGF-I to IGFBP-3 is reduced when the complex becomes cell surface-associated thereby increasing the release of IGF-I for binding to the type-I IGF receptor or insulin receptor for pericellular localisation or for trans-pericellular transport (Wetterau et al., 1999). However the formation of the complex between IGF-I and IGFBP-3 inhibited cell surface binding of IGFBP-3 to Hs578T human breast cancer cells (Oh et al., 1993). These studies highlight the diverse functional role of IGFBP-3 to either promote or inhibit the bioactivity of IGFs and independent actions of IGFBP-3 mediated by cell surface proteins or an unidentified IGFBP-3 receptor.

In *ad libitum* fed guinea pigs most of the variation in organ and tissue weights can be accounted for by the combination of two or three independent covariates. The combination of the ratio of [IGF-I] to [IGFBP-3] and [IGF-II] to [IGFBP-3] plasma concentrations as independent covariates accounts for 73% of the variation in body weight, 80% of the variation in weight gain and 89% of the variation in total uterine weight in *ad libitum* fed guinea pigs (Table 3.23, 3.24).

In feed restricted animals almost all endocrine IGF/IGFBP concentration ratios examined as covariates of tissue and organ weights identified the ratio of [IGF-II] to either [IGFBP-1], [-2] or [-3]. These relationships are weak except for that between [IGF-II] to [IGFBP-1] and weight gain, which is strong (Table 3.23, 3.24). In underfed guinea pigs 73 % of the variation in total uterine weight is accounted for equally by the combination of the independent covariates ratio of [IGF-II] to [IGFBP-2], ratio of [IGF-II] to [IGFBP-1] and ratio of [IGF-I] to [IGFBP-3] in plasma (Table 3.24).

Plasma IGF-II was identified as an independent covariate of placental weight in feed restricted pregnant animals (Table 3.27). As the above relationships between uterine weight and the ratio of the [IGF-II] to either [IGFBP-1] or [IGFBP-2] in plasma are negative, this suggests that high levels of IGFBP-1 and –2, relative to IGF-II, are delivering IGF-II to the placenta to promote its growth. The positive correlation between uterine weight and the ratio of [IGF-I] to [IGFBP-3] in feed restricted animals suggest IGFBP-3 inhibits the access of IGF-I to uterine tissues at this level of nutrition.

The combination of the ratio of [IGF-I] to [IGFBP-1], [-2] and [-3] can account for 89% of the variation in soleus muscle weight in *ad libitum* fed animals (Table 3.24). The ratio of [IGF-I] to either [IGFBP-3] or [IGFBP-4] in combination with the ratio of [IGF-II] to [IGFBP-2] in *ad libitum* fed animals can account for 93% of the variation in thymus weight.

3.4.10 Delivery and actions of endocrine IGFs in pregnancy

IGFBP-3 and IGFBP-2 have a characteristic heparin binding site which could localise associated IGF-I or -II to cells. IGFBP-2 when complexed to either IGF-I or IGF-II has been suggested to undergo a conformational change exposing a glycosaminoglycan-binding domain that can then bind proteoglycans on the cell surface or within the extra-cellular matrix (Arai et al., 1996). Cultured porcine aortic smooth muscle cells secrete a serine protease for IGFBP-2. Coincubation of IGFBP-2 and either IGF-I or IGF-II with media conditioned by porcine aortic smooth muscle cells increased the proteolytic degradation of IGFBP-2 (Gockerman and Clemmons, 1995). Proteolytic activity was more effective when IGFBP-2 was coincubated with IGF-II. In pregnancy IGFBP-2, -3 and -4 are also proteolytically modified. The RGD binding motif in IGFBP-2 may bind to integrin $\alpha_5\beta_1$ (Rauschnabel et al., 1999). IGFBP-2 interaction with this integrin may stimulate secretion of tissue specific proteases ultimately

affecting the affinity of IGFBP-2 for IGFs. This mechanism may be a means of releasing IGF-I from IGFBPs after delivery to tissue making it available to receptors. Epithelial basement membranes such as those present in trophoblasts cells in the placenta are rich in heparan sulfate proteoglycans which can readily concentrate IGFBP-2 and associated IGF-I and -II to these sites (Kreis, 1993).

Delivery of IGF-I to the fetal-maternal interface may have different functions at different stages of pregnancy. Early in pregnancy interactions between IGF-I, IGFBP-1 and/or IGFBP-2, integrin and extra-cellular matrix appear to be involved with cytotrophoblast proliferation and migration (Gibson et al., 2001).

Later in pregnancy when fetal growth places a significant demand on maternal energy resources, IGF-I, IGFBP-1 and IGFBP-2 may act together or independently, increasing maternal supply of glucose and amino acids and/or by altering placental metabolism to provide these substrates for the fetus. Infusion of IGF-I into pregnant sheep at 129-133 days gestation increased placental production and fetal uptake of lactate, while decreasing blood amino nitrogen in both mother and fetus (Liu et al., 1994). Maternal endocrine IGF-I has been suggested to increase carbohydrate availability and affect amino acid metabolism, although increased uptake by the fetus or placenta of those substrates remains to be determined (Harding et al., 1994). Increased circulating levels of IGF-I in pregnancy has also been suggested to regulate glucose transporters GLUT 1 and GLUT 3, which have been identified in the placenta. GLUT 1 is located on the microvillous and basal membrane of the human syncytiotrophoblast whereas GLUT 3 is localized in the vascular epithelium of the placenta and is proposed to be a scavenger removing glucose from blood already partially depleted by GLUT 1 (Illsley, 2000). IGF-I, IGF-II and insulin have been shown to increase 2-deoxyglucose uptake (Kniss et al., 1994) suggesting a mechanism of increasing glucose transport to the placenta and fetus.

3.5 SUMMARY

This study has shown that hepatic IGF-I mRNA is the major source of endocrine IGF-I in pregnancy in guinea pigs. The relationship between hepatic IGF-I mRNA and circulating levels of IGF-I is altered by pregnancy. The abundance of IGF-I mRNA is increased 6 fold and plasma IGF-I is increased 3 fold by pregnancy in *ad libitum* fed guinea pigs. This strongly suggests that pregnancy also increases clearance of IGF-I from blood or alters RNA processing.

The concentrations of IGF-I and/or IGFBP-3 in plasma were major independent covariates of maternal tissue and organ weights including those of the placenta and fetus. This is consistent with the suggestion that endocrine IGF-I has anabolic effects on both maternal and conceptus tissues as well as facilitating the supply of nutrients and substrates required by the growing placenta and fetus and that IGFBP-3 inhibits these actions. IGF-I is the dominant endocrine IGF in pregnancy in the guinea pig while IGF-II is the dominant endocrine IGF in nonpregnant guinea pigs.

The ratio of the [IGFs] to [IGFBPs] in plasma suggested that in pregnant guinea pigs complexes between IGF-I and either IGFBP-2 or IGFBP-1 were major independent determinants of maternal tissue and organ weights while almost all fetal and placental weights were predicted by the ratio of [IGF-I] to [IGFBP-2] in plasma.

These studies suggest that IGFBPs transport and deliver IGF-I to specific target tissues and regulation of both IGF-I and the IGFBPs are important in maternal adaptation for support of the developing conceptus. Because IGF-I regulates IGFBP synthesis and degradation, the

expression of IGF-I in the liver, its major endocrine source in pregnancy, needs to be very tightly regulated.

CHAPTER 4

GENERAL DISCUSSION

GENERAL DISCUSSION

4.1 Effect of pregnancy on IGF-I production

In the present study the relative abundance of IGF-I mRNA in liver was found to be greater than that in parametrial fat, interscapular fat and retroperitoneal fat of guinea pigs. Previous studies (Gosiewska et al., 1994) also found that IGF-I mRNA was expressed more abundantly in liver than bone and cartilage in guinea pigs. In rats IGF-I mRNA is most abundant in liver (Hoyt et al., 1988) whereas in pigs, adipose tissue has the greatest abundance of GH-dependent IGF-I mRNA (Coleman et al., 1994). Liver appears to be the major source of IGF-I in the guinea pig (section 2.3.2).

Specific assays for the measurement of guinea pig IGF-I mRNA and the reference gene guinea pig β-actin were developed and validated for the present study and used to determine the effects of pregnancy and nutrition on hepatic expression of IGF-I. Pregnancy increased the relative abundance of hepatic IGF-I mRNA in *ad libitum* fed guinea pigs. Undernutrition abolished this effect. Most of the variation in the concentration of IGF-I in blood of guinea pigs could be explained by variation in the relative abundance of IGF-I mRNA in liver. This strongly indicates that liver is the primary source of endocrine IGF-I in guinea pigs.

4.2 Regulation of hepatic IGF-I production in pregnancy

Hepatic IGF-I expression in pregnancy is proposed to be regulated by placental hormones such as placental growth hormone and placental lactogen (Caufriez et al., 1990). These polypeptides are structurally similar to pituitary growth hormone, which positively regulates

IGF-I gene expression in well fed nonpregnant adolescent and adult mammals. However nonpregnant guinea pigs continue to grow after hypophysectomy suggesting IGF-I may not be pituitary GH dependent in this species although this remains to be proven directly by treatment with guinea pig GH (Gabrielsson et al., 1990). Keightley *et al* (1996) suggested that the failure of IGF-I to increase in hypophysectomized guinea pigs after treatment with bovine GH or human GH may be related to lack of guinea pig GH receptor recognition by the heterologous hormones used, the impurity of the growth hormone preparations used, the dose and time of administration and/or the age at which GH was given. In the pregnant guinea pig placental growth hormone may be synthesised by the placenta and bind to liver GH receptors to promote IGF-I synthesis. Treatment of guinea pigs with homologous placental lactogen has not been reported.

In nonpregnant guinea pigs all correlations between tissue weights and concentrations of molecular components of the IGF endocrine system contained [IGF-II] alone or as a major component. None of these correlations in nonpregnant guinea pigs contained IGF-I. Therefore somatic growth of the nonpregnant guinea pig appears to be dominated by IGF-II while pregnancy changes this to IGF-I. This finding is unique as in other species the main endocrine IGF covariate of somatic growth is IGF-I.

4.3 Source of endocrine IGF-I in pregnant guinea pigs

The concentration of IGF-I protein in blood plasma was found to be strongly positively correlated with the relative abundance of IGF-I mRNA in liver in the guinea pig. Sixty-eight percent of the variation in plasma IGF-I concentration can be accounted for by the variation in relative abundance of hepatic IGF-I mRNA. Liver therefore appears to be the major source of endocrine IGF-I in guinea pigs. Variation in hepatic abundance of IGF-I mRNA was not associated with variation in plasma IGF-I at concentrations higher than ~850 ng/ml. Viewed

another way, pregnancy increases the abundance of hepatic IGF-I mRNA by 6 fold but increased plasma IGF-I by only 3 fold. This could be explained if the rate of clearance of IGF-I from the circulation in guinea pigs was doubled by pregnancy. Pregnancy has been shown to increase IGF-I clearance in rats (Bastian et al., 1993).

4.4 Potential actions of liver derived endocrine IGF-I in pregnancy

The relative abundance IGF-I mRNA in liver and the concentration of IGF-I in plasma were positively correlated with net body weight, net weight gain, and the weights of retroperitoneal fat, dorsal fat, spleen, thymus, uterus, total fetuses and placentae. Covariate analysis identified maternal plasma IGF-I concentrations as independent covariates of total maternal body weight and weights of liver, uterus, total fetuses and placentae. This indicates that endocrine IGF-I of hepatic origin in pregnancy may have anabolic actions on uterus, fetus and placenta as well as selective tissues in the mother. This view is partly supported by a recent study in which IGF-I treatment of *ad libitum* fed guinea pigs from day 20 to 37 of pregnancy increased fetal and placental weights but not weights of maternal tissues by day 40 of pregnancy [Sohlstrom, 2001 #772

In nonpregnant guinea pigs the abundance of IGF-I mRNA in liver was positively correlated to liver weight only while plasma IGF-I concentration was not related to weight of any tissue suggesting somatic growth of nonpregnant guinea pigs is not dependent on endocrine IGF-I. This is supported by a previous study in which treatment of nonpregnant guinea pigs with IGF-I increased adrenal weight but not that of other tissues (Conlon et al., 1995).

4.5 The effect of pregnancy on the endocrine IGF system in guinea pigs

The concentrations of IGF-I and IGF-II in plasma were positively correlated with almost the same tissue and organ weights in pregnancy except for liver weight as a % of body weight and uterine weight as a % of carcass weight, which were positively correlated to IGF-I only. Covariate analysis showed that plasma IGF-I in pregnancy was a determinant of maternal body weight, maternal weight gain during pregnancy, liver weight, pregnant uterine weight, fetal weight and placental weight. In nonpregnant animals however, IGF-II and not IGF-I was the major determinant of weights of tissues and organs. Pregnancy appears to change the dependence of somatic and organ growth from endocrine IGF-II to IGF-I in the guinea pig. This may also partly explain the lack of growth response of guinea pigs to hypophysectomy, since IGF-II is not directly GH-dependent.

4.6 Role of circulating IGFBP-3 in pregnancy

The concentrations of IGFBP-3 in plasma were unaltered by pregnancy in guinea pigs as detected by western ligand blotting and pregnancy associated IGFBP-3 proteases were not evident in plasma in this species. Plasma IGFBP-3 concentration was positively correlated with the relative abundance of hepatic IGF-I mRNA and the concentrations of IGF-I protein in plasma. Therefore it is possible that hepatic IGFBP-3 expression may be regulated by IGF-I as in other species liver is a major source of endocrine IGFBP-3 (Clemmons, 1991). The concentrations of plasma IGF-I and IGFBP-3 in day 60 pregnant guinea pigs were positively related to total fetal weight, total placental weight and the average weights of fetuses and placentae, suggesting IGFBP-3 might potentially enhance the actions of IGF-I on the placenta and /or fetus. In recent studies, the concentrations of IGF-I and IGFBP-3 in blood of pregnant guinea pigs were found to be positively correlated with placental vascularity, structural maturation and syncytiotrophoblast surface area and thickness (Roberts, 2002). Since

treatment of pregnant guinea pigs with IGF-I increases placental weight (Sohlstrom et al., 2001), cell surface-associated binding of IGFBP-3 may potentiate endocrine IGF-I action by facilitating the delivery of IGF-I to placental type I IGF receptors (Firth et al., 1998;Wetterau et al., 1999).

The concentration of IGFBP-3 in blood plasma in pregnancy was identified as an independent covariate of net body weight, weight gain and the weights of carcass, retroperitoneal fat, dorsal fat, spleen and thymus suggesting IGFBP-3 may have actions independent of those of IGF-I mediated by adherence of IGFBP-3 to cell surface proteins and/or an unidentified IGFBP-3 receptor (Figure 4.1). The correlations between weights of these tissues and [IGFBP-3] alone was much stronger than the corresponding correlations with the ratio of [IGF-I] to [IGFBP-3]. All associations between maternal tissue weights and the ratio of [IGF-I] or [IGF-II] to [IGFBP-3] were very minor and negative. The associations between maternal tissue weights and [IGFBP-3] was equally as strong as that seen with [IGF-I]. In this study a comparison between the concentrations in blood of the independent components of the IGF endocrine system as well as the ratio of the concentrations of IGFs to IGFBPs were evaluated as covariates of the weights of maternal organs. This comparison suggested that growth of retroperitoneal fat, spleen, net body weight, maternal weight gain and carcass weight are determined principally by the concentration of IGFBP-3 in plasma. For some maternal tissues the concentrations of two or more components of the IGF endocrine system were independent covariates which when combined accounted for a greater amount of variation of tissue weights. For example the weight of the spleen is best explained by combination of independent contributions of the concentration of IGFBP-3 and that of IGF-II in plasma.

4.7 Importance of IGFs associated with IGFBPs in the circulation

In this study the ratio of the concentration of IGF-I or IGF-II to that of IGFBP-1, -2, -3 or -4 in plasma were calculated as indicators of the concentrations of binary molecular complexes in blood. In pregnant animals the ratio of [IGF-I] to [IGFBP-2] in plasma was identified as the major independent covariate of maternal body weight, weight gain and weights of carcass, uterine, retroperitoneal fat, gastrointestinal tract, fetuses and placentae. These associations are stronger than those with IGFBP-2 alone and are equally as strong as those with IGF-I alone. This suggests that any actions of IGFBP-2 on these tissues are IGF-I dependent (Figure 4.1).

Weights of other maternal tissues are determined by the ratio of [IGF-I] to [IGFBP-1] in plasma including net body weight, dorsal fat weight, spleen weight, soleus muscle weight, thymus weight, liver weight and average ratio of fetal weight to placental weight. These correlations between the ratio of [IGF-I] to [IGFBP-1] with maternal tissue weights are much stronger than those observed with IGFBP-1 alone and are equally as strong as those for IGF-I alone suggesting any actions of IGFBP-1 on those tissues are also IGF-I dependent.

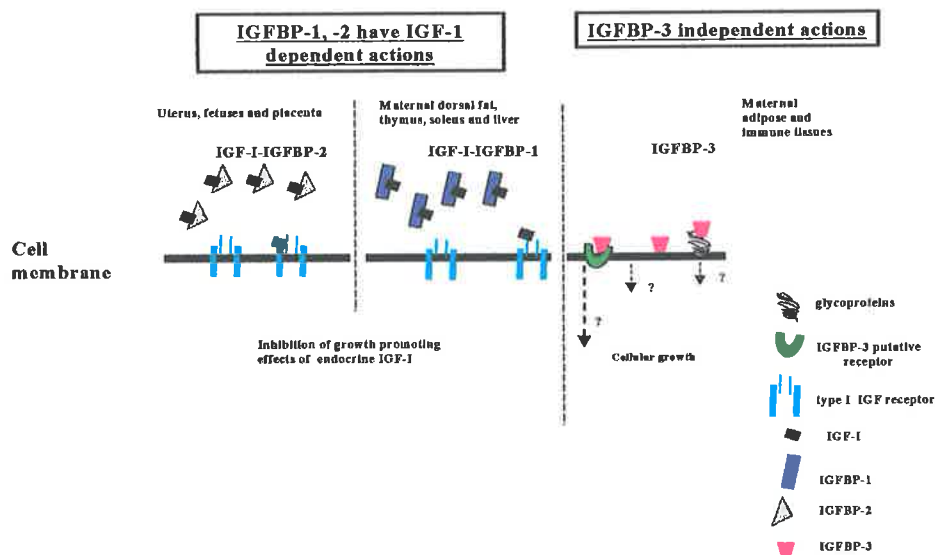


Figure 4.1 Proposed IGFBP-1 and IGFBP-2 IGF dependent and IGFBP-3 independent actions in pregnancy

In the present study the ratio of [IGF-I] to [IGFBP-2] is in plasma a better independent covariate of pregnant uterus weight, total fetal weight and total placental weight at day 60 than that seen with [IGFBP-2] alone. [IGF-I] alone is a better independent correlate of total fetal weight than the ratio of [IGF-I] to [IGFBP-2] or the combination of the ratio of [IGF-I] to [IGFBP-2] and that of [IGF-II] to [IGFBP-2]. [IGF-I] alone and the combination of the ratios of the concentration of [IGF-I] to [IGFBP-2] and [IGF-II] to [IGFBP-2] are similar independent covariates of total placental weight.

The positive relationship between the ratio [IGF-I] to [IGFBP-2] and pregnant uterine weight, total fetal weight and total placental weight suggests IGFBP-2 may inhibit endocrine growth promoting effects of IGF-I on the conceptus in late pregnancy. The increase in IGFBP-2 in feed restricted pregnant guinea pigs may prevent IGF-I from interacting with type I IGF receptor and/or the insulin receptor at the maternal fetal interface. Type I IGF receptors have been identified in the second and third trimester of human pregnancy in the chorionic villi, chorionic plate, basal plate and fetal membranes (Han et al., 1996). Location of the type I IGF receptors have not been reported in the guinea pig placenta as yet.

Alternatively an increase in the ratio of [IGF-I] to [IGFBP-2] in pregnant guinea pigs, an indicator of the concentration of this binary complex, may reflect a potential increase in this circulating reservoir of endocrine IGF-I. Studies have demonstrated that binding of IGF-I to IGFBP-2 facilitates proteolysis of IGFBP-2 by exposing a proteolytic cleavage site (Gockerman and Clemmons, 1995), potentially increasing the availability of IGF-I to tissue receptors. Binding of IGF-II to IGFBP-2 also increases proteolysis of IGFBP-2.

4.8 Nutrition and circulating IGFBP-1 in pregnancy

In this study the ratio of the concentration of IGF-I to IGFBP-1 in the maternal circulation is an independent covariate of the weights of dorsal fat, spleen, thymus, soleus muscle, liver and net maternal body weight and these associations are stronger than that of IGFBP-1 alone. In contrast in feed restricted mothers the ratio of [IGF-II] to either [IGFBP-1] or [IGFBP-2] are associated with weights of maternal tissues and none of these associations contain IGF-I as a covariate. The positive associations seen for the weights of these tissues suggest IGFBP-1 and/or IGFBP-2 inhibits growth promoting actions of IGF-I or -II on these maternal tissues depending on nutritional status.

IGFBP-1 has been suggested to have a potential role at the maternal-fetal interface regulating placental and fetal growth (Gibson et al., 2001). This is a complex interaction where IGFBP-1 either inhibits the bioavailability of IGF-I or IGF-II or conversely increases the potency of the growth factor by facilitated interaction with type I IGF receptor and/or type II IGF receptor by IGFBP-1 interactions with $\alpha_5\beta_1$ integrin. In this study in feed restricted animals the ratio of [IGF-I] to [IGFBP-1] in plasma was found to be a positive covariate of the number of fetuses per dam, total fetal weight and total placental weight. This therefore suggests that IGFBP-1 in the undernourished guinea pig at day 60 of pregnancy may inhibit growth promoting properties of endocrine IGF-I on dorsal fat, soleus muscle, fetuses and placentae. A proposed inhibitory action of IGFBP-1 on fetal and placental tissues in pregnant guinea pigs is supported by observations in human pregnancy where maternal plasma IGFBP-1 levels are negatively related to birth weight (Iwashita et al., 1992), placental weight and maternal weight (Hills et al., 1996). Also, intrauterine growth retardation is associated with increased maternal and fetal plasma concentrations of IGFBP-1 in humans (Hills et al., 1996) and mothers of small for gestational age babies have higher plasma levels of IGFBP-1 than those with average for gestational age babies. Furthermore maternal IGFBP-1 are 5-fold

higher and plasma IGF-I is 2-fold lower in preeclamptic subjects with growth retarded fetuses (Giudice et al., 1997).

4.9 Summary of independent covariates of tissue weights

Individual components of the IGF endocrine system were identified as major positive correlates of pregnant tissue weights (Table 4.1). Plasma concentration of IGF-I was positively correlated with weights of fetuses, placentae and some maternal tissues while the concentration of IGFBP-3 was positively correlated with another group of tissues, which are different from those whose weights are related to endocrine IGF-I concentration. Soleus muscle was the only tissue whose weight was positively correlated with endocrine IGF-II concentration. The ratio of the concentration of IGF-I to that of IGFBP-2 was a positive correlate of weights of several maternal tissues. This suggests that complexes formed between IGF-I and IGFBP-2 may have different biological roles at different tissue sites. The ratio [IGF-I]/[IGFBP-1] was also correlated with weights of certain tissues in pregnancy but these are different tissues from those whose weights are correlated with [IGF-I]/[IGFBP-2]. For example, weight of retroperitoneal fat is positively correlated with [IGF-I]/[IGFBP-2] whereas, weight of dorsal fat is positively correlated with [IGF-I]/[IGFBP-1]. These results suggest that different fat sites are differentially regulated in pregnancy and this is IGFBP dependent. Alternatively pregnancy may alter the expression of different IGFBPs in different tissues. All of the IGF endocrine concentration ratios that correlate with weights of tissues in pregnancy contain the concentration of IGF-I while none contain that of IGF-II. When comparing the concentration of individual components of the IGF system to those of the ratios of [IGFs]/[IGFBPs] that were identified as independent covariates of tissue weights many of the correlations are IGF-I dependent although some correlations have suggested IGFBP-3 has independent actions on some maternal tissues. This suggests there may be actions of IGFBP-

3 mediated through putative IGFBP-3 receptors, or by IGFBP-3 cell surface-association, which may potentiate the actions of endocrine IGF-I.

Table 4.1 Covariates of tissue weights in pregnant guinea pigs.

Individual components of the IGF system as independent covariates	The ratio of [IGFs]/[IGFBPs] as independent covariates	Pregnant tissue weight
(+) IGF-I	(+) IGF-I/IGFBP-2	Maternal body weight Fetuses Placentae
(+) IGFBP-3	(+) IGF-I/IGFBP-2	Weight gain Carcass Retroperitoneal fat
(-) IGF-I	(-) IGF-I/IGFBP-2	Gastrointestinal tract
(+) IGFBP-3	(+) IGF-I/IGFBP-1	Net body weight Dorsal fat Spleen Thymus
(+) IGF-I	(+) IGF-I/IGFBP-1	Liver
(+) IGF-II	(+) IGF-I/IGFBP-1	Soleus muscle

In nonpregnant guinea pigs plasma IGF-II concentrationwas identified as an individual correlate of almost all tissues studied (Table 4.2). The concentration of IGFBP-3 was identified as an independent correlate of weights of retroperitoneal fat and soleus muscle in nonpregnant guinea pigs and these tissues are different from those whose weights in pregnant animals are related to concentration of endocrine IGFBP-3. In contrast to the correlations with tissue weights seen in the pregnant guinea pig all ratios contained [IGF-II] and none contained [IGF-I] in the nonpregnant animal. In nonpregnant guinea pigs the ratio of [IGF-

II]/[IGFBP-2] was a positive correlate of the majority of tissues studied. When comparing the individual IGF endocrine component concentrations with those of the ratio of [IGFs]/[IGFBPs] that were identified as independent covariates, IGFBP-3 may have independent actions on both retroperitoneal fat and soleus muscle. The actions of IGF-II:IGFBP complexes on other tissues appear to be IGF-II dependent.

Table 4.2 Covariates of tissue weights in nonpregnant guinea pigs.

Individual components of the IGF system as covariates	The ratio of [IGFs]/[IGFBPs] as independent covariates	Nonpregnant tissue weights
(+) IGF-II	(+) IGF-II/IGFBP-2	Total body Net body weight Weight gain Carcass Dorsal fat Spleen Thymus Liver
(+) IGFBP-3	(+) IGF-II/IGFBP-2	Retroperitoneal fat Soleus muscle
(+) IGF-II	(+) IGF-II/IGFBP-4	Uterus
	(-) IGF-II/IGFBP-1	Gastrointestinal tract

4.10 Future Directions

The studies in this thesis has established the liver as the major source of increased circulating IGF-I in pregnancy in guinea pigs. Hepatic abundance of IGF-I mRNA was increased by pregnancy in *ad libitum* fed animals. The quantitative relationship between relative abundance of hepatic IGF-I mRNA and plasma concentrations of IGF-I is positive although not linear. Future studies need to identify the mechanism by which pregnancy increases expression of IGF-I in liver, and identify specific IGF-I mRNA transcripts that are primarily translated into IGF-I protein in pregnancy.

In this study many of the associations between plasma IGF-I concentration with maternal, fetal and placental tissue weights and organ weights in pregnancy are strong and positive suggesting a major role for endocrine IGF-I in promoting growth. The concentration of IGFs in pregnancy and relationships with specific tissue weights suggest IGF-I may be a dominant growth factor and is important for maternal adaptation to pregnancy. Estimation of the interactions between IGFs and IGFBPs suggests pregnancy alters the binary molecular complexes and they are tissue specific in their actions. IGFBPs may deliver IGF-I to some tissues and prevent their action on others. The development of diagnostic assays for the measurement of circulating complexes between IGFs and IGFBP-1, IGFBP-2 and IGFBP-3 and the phosphorylation status of IGFBP-1 in pregnant blood may be of clinical value.

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