EFFECTS OF IGF-I OR LR^3IGF-I INFUSION ON COMPONENTS OF THE GH/IGF-I AXIS IN PIGS

by

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ADDENDUM

Page 5 line 8: the Jansen *et al.*, 1983 reference is incorrect and should be Baxter, 1988.

Page 20 line 7: should read "insulin receptor"

Page 29 line 4: should read "restored".

Page 32 line 21: the statement "The acid labile subunit blocks binding of IGF-I and II to the IGF receptor and has a higher affinity binding for IGF-I than does the type I IGF receptor (Francis *et al.*, 1988; Gopinath *et al.*, 1989)." is incorrect and refers to the acid stable subunit (IGFBP-3) not acid-labile subunit.

Page 35 line 15: should read "Alternative splicing of the human GH gene..."

Page 37 line 9: Although Weeden *et al.*, (1993) showed that GH treatment increases average daily weight gain and feed : gain ratio in pigs, the efficacy of GH treatment in pigs was demonstrated as early as 1986 (Etherton *et al.*, 1986).

Full reference: Etherton T.D., Wiggins J.P., Chung C.S., Evock C.M., Rebhun J.F. and Walton P.E. (1986) Stimulation of pig growth performance by porcine growth hormone and growth hormone-releasing factor. *Journal of Animal Science* **63**, 1389-1399

Page 38 line 14: the Evock *et al.*, 1988 reference is incorrect and should be Goodband *et al.*, 1993. This reference is also incorrectly cited on page 146 line 12.

Full reference: Goodband R.D., Hines R.H., Nelssen J.L., Kropf D.H. and Schricker B.R. (1993) Porcine somatotropin and dietary lysine influence bone mineralization and mechanical properties of bones in finishing swine. *Journal of Animal Science* **71**, 673-678

Page 67 Table 3.5: AUC is an abbreviation for area under curve.

Page 101 line 12: should read "alluded"

Page 130 line 2: The anterior vena cava was cannulated with two indwelling catheters.

Page 146 line 19: should read "diseased"

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ABSTRACT

Insulin-like growth factor-I (IGF-I) promotes protein and DNA synthesis, inhibits protein breakdown and enhances growth in normal rats. A variant of IGF-I, Long R^3IGF-I (LR^3IGF-I), that binds poorly to IGF binding proteins is 10-fold more potent than IGF-I *in vitro*. LR^3IGF-I is even more anabolic in rats than IGF-I. In pigs, LR^3IGF-I inhibits growth, while IGF-I has no effect on growth performance.

The aim of this project was to determine why LR^3IGF-I has such divergent effects in two different species. One hypothesis is that LR^3IGF-I may have different effects on the GH/IGF-I axis depending on the species. I therefore investigated the endocrine regulation of IGF-I and IGF binding protein-3 (IGFBP-3) in the pig and determined the effects of IGF-I and LR^3IGF-I treatment on porcine IGF-I and IGFBP-3 expression at the gene and protein level.

In the first study, the effects of continuous 4 day infusion of IGF-I or LR^3IGF-I alone and in combination with porcine growth hormone (pGH) on growth performance and plasma hormone levels was investigated. This study examined whether the poor growth response that had been previously observed was due to hypoglycaemia induced by the IGF peptides and whether exogenous administration of IGF-I or LR^3IGF-I affected components of the GH/IGF-I axis. This was determined by measuring plasma IGF-I, IGFBP-3 and GH levels. LR^3IGF-I significantly decreased average daily gain when compared to animals receiving IGF-I, and decreased feed intake. Although co-administration with pGH did return growth performance of LR^3IGF-I treated pigs to normal, there were no synergistic actions on growth performance between the two peptides. Neither IGF-I or LR^3IGF-I when administered as a chronic infusion affected plasma glucose levels, indicating that the poor growth response observed in these animals was not due to hypoglycaemia. Plasma insulin levels were reduced by IGF-I and LR^3IGF-I, consistent with similar studies in man and sheep where IGF-I treatment suppressed plasma insulin levels. IGF-I and LR^3IGF-I treatment suppressed average plasma GH levels, suggesting that the peptides were acting on the GH/IGF-I axis to inhibit GH production or secretion from the pituitary. LR^3IGF-I also blunted the magnitude of the pulsatile expression of GH by decreasing the area under the GH peaks. Similar effects of IGF-I on GH secretion have also been reported in man and sheep. In the present study, the decrease in plasma GH was associated with a decrease in plasma IGF-I and IGFBP-3 in LR^3IGF-I treated animals. Co-administration with pGH was not able to return plasma levels of IGF-I and IGFBP-3 to normal. In the rat, LR^3IGF-I treatment has no effect on plasma IGF-I levels, and plasma IGFBP-3 levels are increased in these animals. These results suggest that in the pig, LR^3IGF-I inhibits

components of the GH/IGF-I axis and this is responsible for the poor growth response seen in pigs.

Following on from these findings, I postulated that the decrease in plasma IGF-I and IGFBP-3 were due to a suppression in gene transcription. In order to determine this, it was first necessary to establish in which porcine tissues IGF-I and IGFBP-3 gene expression was regulated by GH. Female pigs of the same age as used in the previous study were treated with 70 μg/kg/day porcine GH for 5 days, sacrificed and samples of liver, kidney, muscle, stomach and small intestine were snap frozen in liquid nitrogen and stored at -80^0C. Total RNA was extracted and expression of IGFBP-3 mRNA was measured by RNase protection assay using a previously constructed porcine IGFBP-3 probe. For IGF-I analysis, expression of IGF-I *class 1* and *class 2* transcripts was quantitated in a similar manner using porcine specific probes. In the liver, pGH treatment significantly increased IGF-I *class 1*, *class 2* and IGFBP-3 gene expression. In the kidney, there were no changes in IGF-I *class 1* gene expression and IGF-I *class 2* mRNA could not be detected. Kidney IGFBP-3 gene expression was significantly increased. None of the other tissues examined showed changes in IGF-I or IGFBP-3 gene expression in response to pGH treatment. This is the first study to investigate GH regulated gene expression of IGFBP-3 in different porcine tissues. GH dependent expression of the two different IGF-I transcripts in pigs was only reported in August 1996 and was confined to mRNA analysis in liver, adipose tissue and longissimus dorsi and semitendinosus muscle. IGF-I *class* 1 expression has been shown to increase with GH treatment in liver, adipose tissue, semitendinosus but not longissimus dorsi muscle. From the present study it was concluded that in the liver, IGF-I *class 1*, *class 2* and IGFBP-3 gene expression are increased with pGH treatment, and IGFBP-3 mRNA is increased in the kidneys of pGH treated animals.

In the next part of the project, pigs were intravenously infused with IGF-I or LR^3IGF-I for 5 days to determine their effect on endogenous IGF-I and IGFBP-3 gene expression in liver and kidney. In agreement with the earlier study, LR^3IGF-I treatment decreased plasma IGF-I and IGFBP-3 concentrations. Both IGF-I and LR^3IGF-I treatment decreased liver IGF-I *class 2* but not IGF-I *class 1* gene expression. Liver IGFBP-3 and kidney IGF-I and IGFBP-3 gene expression were not affected by either IGF-I or LR^3IGF-I treatment. It is most likely that IGF-I *class 2* mRNA expression is more sensitive to changes in plasma GH status than IGF-I *class 1* mRNA, since only expression of *class 2* transcripts was affected by IGF-I or LR^3IGF-I treatment. Expression of liver IGF-I *class 2* mRNA has been shown to be closely correlated to plasma IGF-I levels. The reduction in liver IGF-I *class 2* mRNA is therefore likely to be a major contributor to the reduction in plasma IGF-I levels. Although plasma IGFBP-3 levels were significantly reduced in LR^3IGF-I treated animals, liver and kidney IGFBP-3 mRNA expression

was not affected. The decrease in plasma IGFBP-3 may therefore be a result of post translational modifications such as mRNA instability or IGFBP-3 protease activity. The observed increases in kidney IGFBP-3 expression during pGH treatment could be a result of increased IGF-I in the circulation binding to kidney IGF type 1 receptors and increasing IGFBP-3 expression.

The final study was designed to investigate if combination treatment of IGF-I or LR^{3}IGF-I with pGH for 14 days was able to restore liver IGF-I gene expression. Pigs receiving LR^{3}IGF-I treatment alone or in combination with pGH lost their appetite and refused to eat. Two pigs (one from the LR^{3}IGF-I group, the other from the LR^{3}IGF-I + pGH group) had to be euthanised after 7 and 9 days of treatment. Another LR^{3}IGF-I treated pig was found dead in its pen on day 10 of treatment. This made data analysis for this group not possible. IGF-I treatment alone and in combination with pGH had similar effects on plasma IGF-I and IGFBP-3 levels as described in previous studies. In this study, pGH treatment alone significantly increased liver IGF-I *class 2* but not *class 1* mRNA. The dose administered in this trial was less than half that administered in the previous experiment, and confirms that IGF-I *class 2* mRNA expression is more sensitive to GH status than IGF-I *class 1* mRNA expression. IGF-I treatment for 14 days significantly decreased liver IGF-I *class 2* mRNA, which were restored to normal when pGH was administered in combination with IGF-I. There were no effects on liver and kidney IGF-I *class 1* or IGFBP-3 mRNA levels for any of the treatments. Co-administration of pGH with IGF-I can therefore compensate for some of the effects IGF-I treatment has on components of the GH/IGF-I axis. It is unlikely that pGH has the same effects on IGF-I mRNA expression in pigs treated with LR^{3}IGF-I, since plasma IGF-I and IGFBP-3 levels are reduced even when the two hormones are given in combination.

This project has shown that LR^{3}IGF-I treatment in pigs reduces average daily gain and feed intake. This is not due to the hypoglycaemic effects often associated with IGF treatment, but rather a result of reduced plasma GH levels. This reduction in plasma GH is achieved by either direct inhibition of GH mRNA expression or secretion from the pituitary and/or via a long-loop feedback inhibition at the hypothalamus by regulating somatostatin and/or growth hormone releasing hormone. The reduction in plasma GH affects components of the GH/IGF-I axis, which leads to reduction in IGF-I gene and protein expression as well as reduced plasma IGFBP-3 levels.

The different responses to LR^{3}IGF-I treatment between rats and pigs may be due to different levels of GH sensitivity between the species. Growth performance in the rat can be increased by GH at doses of 1 mg/kg/day, without altering plasma IGF-I levels, while in pigs doses as low as 30 μg/kg/day are sufficient to improve growth performance and elevate plasma

IGF-I levels. Since most of the growth promoting actions of GH are mediated by IGF-I, then this suggests that in the rat, IGF-I expression is not very sensitive to GH status. If IGF peptides do reduce plasma levels of GH in the rat as they do in the pig, the growth promoting actions of IGF-I are not impaired in rats, since IGF-I levels are not reduced with IGF-I treatment in this species.

STATEMENT OF ORIGINALITY

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

Vera Dunaiski

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PUBLICATIONS

Dunaiski V., Dunshea F.R., Walton P.E. and Goddard C. Long [R^3] insulin-like growth factor-I (LR^3IGF-I) reduces growth, plasma growth hormone, IGFBP-3 and endogenous IGF-I concentration in pigs. *Journal of Endocrinology* (submitted)

Robertson J.G., Walton P.E., Dunshea F., Dunaiski V., Ballard F.J. and Belford D. (1997) Growth hormone but not insulin-like growth factor-I improves wound strength in pigs. *Wound Repair and Regeneration* (in press)

Abstracts of thesis research presented at scientific meetings

Dunaiski V., Dunshea F.R., Clarke I., Owens P.C. and Walton P.E. (1994) Endocrine regulation of growth hormone secretion by IGF-I and IGF-I analogues in pigs. *Proceedings of the Endocrine Society of Australia* 37, Abstract 123

Dunaiski V, Dunshea F.R., Clarke I., Goddard C., Owens P.C. and Walton P.E. (1995) Endocrine regulation of somatotropin secretion by IGF-I and IGF-I analogues in pigs. *Manipulating Pig Production V,* 86

Dunaiski V., Dunshea F.R. and Goddard C. (1996) Regulation of insulin-like growth factor-I (IGF-I) and IGFBP-3 gene expression in finisher pigs. *Proceedings of the Endocrine Society of Australia* 39, Abstract 5

ABBREVIATIONS

Amino acid symbols and abbreviations

three letter abbreviation	one letter symbol	amino acid
Ala	A	alanine
Arg	R	arginine
Asn	N	asparagine
Asp	D	aspartic acid
Cys	C	cysteine
Gln	Q	glutamine
Glu	E	glutamic acid
Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Leu	L	leucine
Lys	K	lysine
Met	M	methionine
Phe	F	phenylalanine
Pro	P	proline
Ser	S	serine
Thr	T	threonine
Trp	W	tryptophan
Tyr	Y	tyrosine
Val	V	valine

ADG	average daily gain
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	di-deoxyadenosine triphosphate

ddCTP	di-deoxycytidine triphosphate
ddGTP	di-deoxyguanosine triphosphate
ddTTP	di-deoxythymidine triphosphate
DEP-C	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
x *g*	times the force of gravity
GRF	growth hormone releasing hormone
h	hours
HCl	hydrochloric acid
H_2O	water
icv	intracebroventricular
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
kb	kilobases
kDa	kilodaltons
kg	kilograms
l	litre
LiCl	lithium chloride
M	molar
mg	milligrams
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MSA	multiplication-stimulating activity
NaCl	sodium chloride
$Na\text{-}I^{125}$	sodium iodide

NaOH	sodium hydroxide
ng	nanograms
NSILA	non-suppressible insulin-like activity
RNA	ribonucleic acid
P	probability
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
pGH	porcine growth hormone
RIA	radioimmunoassay
RGD	receptor recognition sequence
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SFA	sulphation factor
TCA	trichloroacetic acid
TGF	transforming growth factor
UTR	untranslated region
μg	micrograms
μl	microlitres
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

In rats, growth performance can be enhanced in a dose dependent manner by subcutaneous administration of IGF-I or LR^{3}IGF-I, a more potent analogue of IGF-I (Tomas *et al.*, 1993). Similar results are also produced *in vitro,* with LR^{3}IGF-I being ten times more potent than IGF-I in promoting cellular proliferation as measured by DNA and protein synthesis assays (Francis *et al.*, 1992). In contrast, IGF-I has little effect, and LR^{3}IGF-I reduces average daily weight gain in pigs (Walton *et al.*, 1994).

The purpose of this work has been to investigate the effects of IGF-I and LR^{3}IGF-I infusion in pigs on components of the GH/IGF-I axis. Results obtained from this study are compared with analogous rat experiments from the literature in an effort to explain the different growth responses observed between rats and pigs. The information presented here represents a review of the knowledge in the field of IGFs and their endocrine interactions. Included for completeness is a relevant background on IGF receptors and the IGF binding proteins. This review covers the literature to December 1993.

1.2 Historical Aspects

The IGF peptides were first discovered as three separate biological activities in serum. Sulphation factor (SFA), nonsuppressible insulin-like activity (NSILA) and multiplication-stimulating activity (MSA).

Sulphation factor was identified when it was discovered that GH stimulates the uptake of ^{35}S into cartilage from hypophysectomized rats *in vivo,* but not *in vitro*. This lead to the proposal that GH might be acting through an intermediate substance in the serum. This was termed "sulphation factor" (Salmon and Daughaday, 1957). When it was discovered that sulphation factor was able to stimulate the uptake of ^{3}H thymidine into DNA (Daughaday and Reeder 1966) and cause a non-suppressible insulin-like effect in adipose tissue (Hall and Uthne, 1971), it became clear that the name "sulphation factor" had become too restrictive. In 1972 a number of investigators proposed "somatomedin" as an alternative name, linking its hormonal relationship to somatotropin (or GH), and medin indicating that it is an intermediary in GH action. An acidic form of the sulphation factor was termed somatomedin A and the basic sulphation factor was called somatomedin C.

NSILA was discovered when in 1962 it became evident that serum contained insulin-like activity, of which only a small portion was suppressible by insulin antibodies (Leonards

and Landau, 1962). This unaccounted for insulin-like activity was termed NSILA. This factor was 20 times more potent than insulin in stimulating ^{3}H thymidine uptake (Morell and Froesch, 1973) and was shown to be able to stimulate sulphate uptake in rat and chick cartilage (Zing & Froesch, 1973). This led to the suggestion that NSILAs and sulphation factor may be the same molecules in serum.

A third biological activity, MSA was identified in the conditioned medium of rat liver cells as the agent that enabled this cell line to grow in the absence of serum and stimulate DNA synthesis in chick embryo fibroblasts (Dulak and Temin, 1973).

In 1976, Rinderknecht and Humbel (1976) reported finding two species with nonsuppressible insulin-like cell-growth-promoting activities and reported their full amino acid sequences by 1978 (Rinderknecht and Humbel, 1978a; 1978b). These peptides became known as IGF-I and IGF-II. They are single-chain polypeptides sharing identical residues at 45 positions, giving a sequence homology of 62%.

The nomenclature for the IGF peptides was resolved when somatomedins A and C and NSILA-I/IGF-I were found to be identical (Klapper *et al.*, 1983) while NSILA-II/IGF-II and MSA were found to be 93% homologous. MSA was identified as rat IGF-II (Marquardt and Todaro, 1981) while NSILA-II was of human origin.

IGF-I cDNA clones were isolated from a human adult liver library in 1983 (Jansen *et al.,* 1983) and the gene was mapped to the long arm of chromosome 12 (Brisenden *et al.*, 1984; Tricoli *et al.*, 1984). Isolation of the human IGF-II cDNA (Bell *et al.*, 1984; Jansen *et al.*, 1985) allowed the IGF-II gene to be mapped to the short arm of chromosome 11 (Brisenden *et al.*, 1984; Tricoli *et al.*, 1984), closely linked to the insulin and tyrosine hydroxylase loci (O'Malley & Rotwein, 1988).

1.3 IGF-I Peptide and Gene Structure and Expression

To date, the human and rat IGF-I genes have been the most extensively studied. In these species, IGF-I is encoded by a single copy gene which, when transcribed, gives rise to a variety of different mRNA species through the use of multiple leader exons and transcription start sites, differential splicing involving exon 5 and the presence of multiple polyadenylation sites in exon 6. Different pre-protein products are generated by differential splicing of the mRNA and further processing occurs when the translated pre-prohormone is cleaved to yield the mature protein.

1.3.1 Peptide structure

It is generally accepted that all mammals and probably all vertebrates express IGFs. The structure and cDNA sequence of the IGF-I gene has been determined in a number of species including man (Rotwein *et al.*, 1986), rat (Shimatsu & Rotwein, 1987), mouse (Bell et al., 1986), pig (Müller & Brem, 1990), sheep (Dickson *et al.*, 1991), chicken (Kajimoto & Rotwein, 1991), salmon (Kavsan *et al.*, 1993), cow (Fotsis *et al.*, 1990), guinea pig (Bell *et al.*, 1990) and *Xenopus* (Shuldiner *et al.*, 1990). The amino acid sequence of mature IGF-I is identical in man, cow, pig, and guinea pig. Sheep IGF-I differs from these by one residue and three residues in the rat and four in the mouse differ in the B, C and D domains, while chicken, frog and salmon show an increase in divergence from the human sequence. Generally, the A domain is the most conserved, with non-homologous residues residing mainly in the B and C domains (Figure 1.1).

Figure 1.1:

Comparison of the amino acid sequence for mature IGF-I in different species

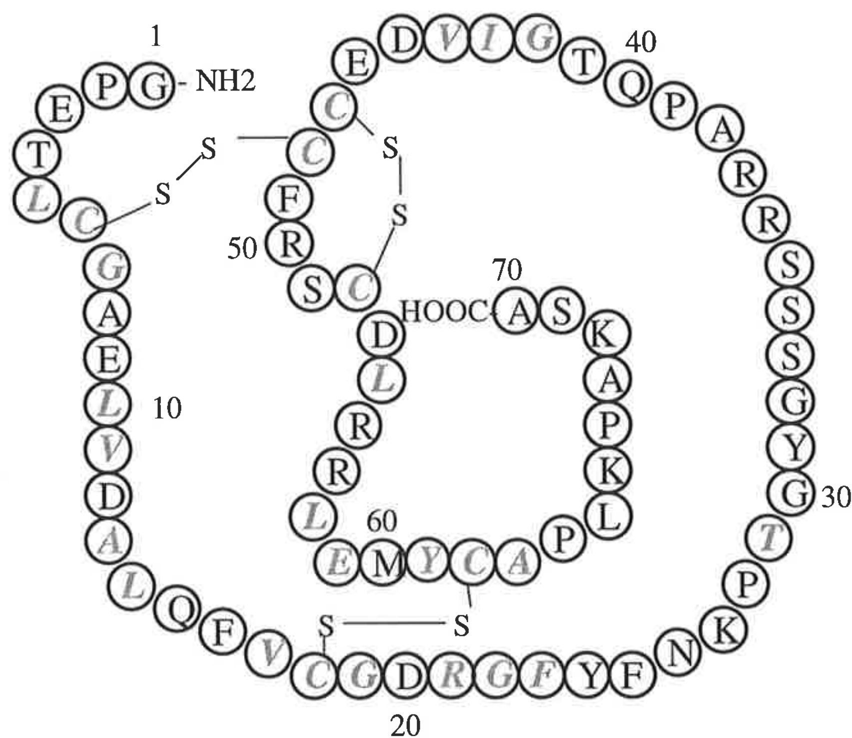
```
                                 B domain                       C domain
Human:       G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T   G Y G S S S R R A P Q T
Pig:         - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   - - - - - - - - - - - -
Cow:         - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   - - - - - - - - - - - -
Sheep:       - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   - - - - - - - - - - - -
Guinea pig:  - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   - - - - - - - - - - - -
Rat:         - - - - - - - - - - - - - - - - - - - P - - - - - - - - -   - - - - - I - - - - - -
Mouse:       - - - - - - - - - - - - - - - - - - - P - - - - - - - - -   - - - - - I - - - - - -
Chicken:     - - - - - - - - - - - - - - - - - - - - - - - - - S - - -   - - - - - - - - L H H K
Frog:        - - - - - - - - - - - - - T - - - - - - - - - - - S - - -   - - - N N - - - S H H R
Salmon:      - - - - - - - - - - - - - T - - - - - - E - - - - S - - -   - - - P - - - - S H N R

                      A domain                          D domain
Human:       G I V D E C C F R S C D L R R L E M Y C A   P L K P A K S A
Pig:         - - - - - - - - - - - - - - - - - - - - -   - - - - - - - -
Cow:         - - - - - - - - - - - - - - - - - - - - -   - - - - - - - -
Sheep:       - - - - - - - - - - - - - - - - - - - - -   - - - A - - - -
Guinea pig:  - - - - - - - - - - - - - - - - - - - - -   - - - - - - - -
Rat:         - - - - - - - - - - - - - - - - - - - - -   - - - - T - - -
Mouse:       - - - - - - - - - - - - - - - - - - - - -   - - - - T - A -
Chicken:     - - - - - - - - Q - - - - - - - - - - - -   - I - - P - - -
Frog:        - - - - - - - - Q - - - F - - - - - - - -   - A - - - - - -
Salmon:      - - - - - - - - Q - - E - - - - - - - - -   - V - S G - A -
```

Dashes indicate amino acids identical to human sequence and domains are indicated above the sequence. (Data was adapted from Ward and Elliss, 1992).

IGF-I is structurally homologous to pro-insulin (Figure 1.2). Insulin exists as a single chain dimer connected by disulphide bridges. Its precursor, proinsulin is produced as a single chain with a connecting C-peptide that links the A and B chains. The C peptide is cleaved to generate the mature insulin molecule (Murray-Rust *et al.*, 1992). The IGFs are single chain peptides. The A and B domains, which share sequence homology with the A and B chains of insulin, are linked via the C domain which is not cleaved in the mature protein. The E domain of the IGFs is only present in the prohormone, and is cleaved when the protein is secreted (Jansen *et al.*, 1983).

Figure 1.2:

Structure of human IGF-I



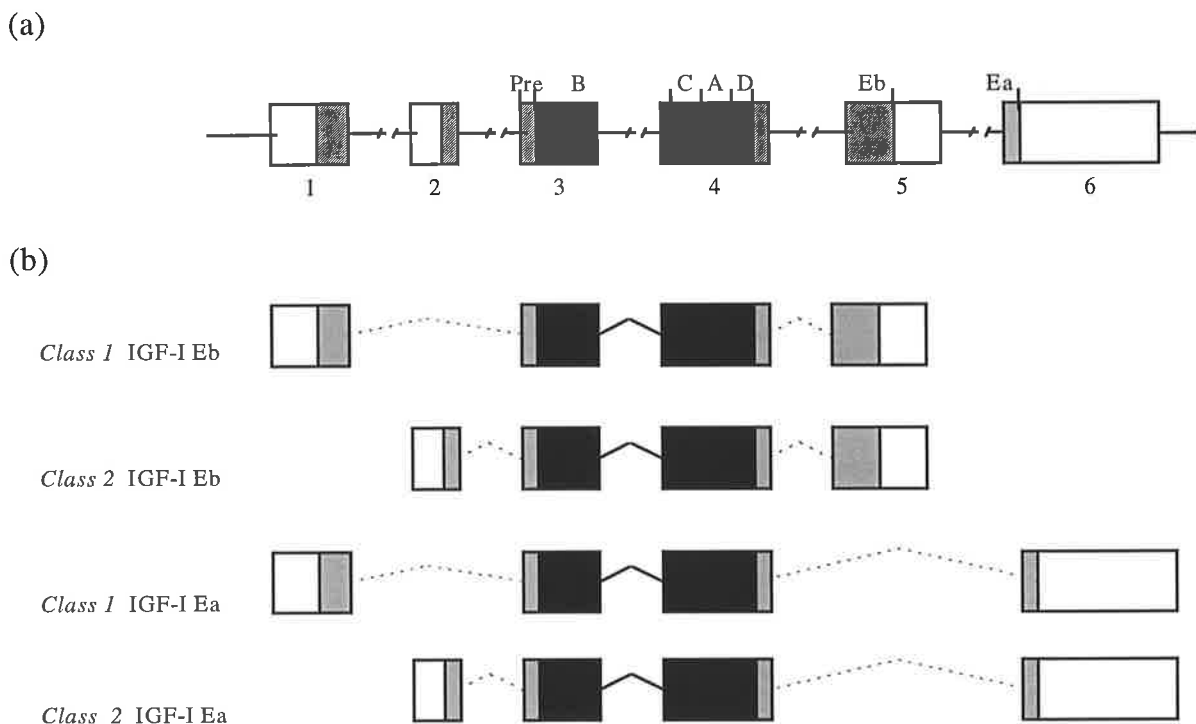
Amino acids identical to insulin are indicated in grey. The B domain extends from amino acids 1 to 29; the C domain from 30 to 41, the A domain from 42 to 62 and the D domain from 63 to 70. Di-sulphide bridges between cysteine residues are indicated.

1.3.2 Gene structure

The human IGF-I gene consists of 6 exons (Adamo *et al.*, 1994) spanning an area of over 90 kilo bases (kb). IGF-I is synthesised as a large precursor molecule which is then processed at both the amino terminus (the signal peptide) and the carboxyl terminus (E domain) to generate a mature 70 amino acid IGF-I protein. The first two exons of the IGF-I gene encode the 5' untranslated region (UTR) and the amino terminus of the signal peptide. The third exon encodes the remainder of the signal peptide and the amino terminus of the B domain. This is followed by sequences coding for the remainder of the B domain, the C, A and D domains and the first part of the terminal E peptide in the fourth exon. Exons 5 and 6 encode alternative forms of the E peptide (Eb or Ea respectively) and a long untranslated region (3'-UTR) containing multiple polyadenylation sites completes exon 6 (Figure 1.3). This architecture of the IGF-I gene is fundamentally conserved across many species.

Figure 1.3

Organisation of the human IGF-I gene and its different mRNA species



The human IGF-I gene (a). Exons are represented as boxes and the area encoding the mature IGF-I sequence is indicated in the solid boxes. Coding sequences for the prohormone are indicated as hatched boxes and untranslated sequences are represented as open boxes. Splicing between alternatively used exons is indicated by the broken lines (b).

The rat IGF-I gene is analogous to the human gene. It is transcribed as two distinct mRNAs containing either leader exon 1 or leader exon 2. Splicing variants at the 3' end of the gene are also similar to that found for the human IGF-I gene.

In the pig, the known coding sequence of IGF-I so far only extends to the 3' untranslated region of exon 5 (Figure 1.4). Comparison of nucleotide sequences for the exon 1 leader peptide, the mature IGF-I protein in exons 3 and 4, exon 5 E peptide and untranslated region show 79%, 86%, 88% and 64% homology between pig, rat and human (Tavakkol *et al* , 1988). As in the rat and human, two types of IGF-I transcripts are generated in the pig by the use of different leader sequences in either exon 1 or exon 2.

Figure 1.4:

Coding sequence of exons 1 to 5 of the porcine IGF-I gene

(exon 1) ....ATGGGAAAAA TCAGCAGTCT TCCAACCCAA TTATTTAAGT
GCTGCTTTTG TGATTTCTTG AAG....(exon 2)....ATGGTTACAC CTACA
(exon 3) ... GTAAAGATGC ACATCACATC CTCTTCGCAT CTCTTCTACT
TGGCCCTGTG CTTGCTCTCC TTCACCAGCT CTGCCACGGC TGGACCTGAG
ACCCTCTGTG GGACGAACGA GAGGAAGTGG TCGAGACGGT GCGACTGGGG
ACTCTGGGAG ACACCCGCTG AGCTGGTGGA CGCTCTTCAG TTCGTGTGCG
GAGACAGGGG CTTTTATTTC .... (exon 4).......AACAAGCCCA CAGGGTACGG
CTCCAGCAGT CGGAGGGCGC CACAGACGGG CATCGTGGAT GAGTGCTGCT
TCCGGAGCTG TGATCTGAGG AGGCTGGAGA TGTACTGTGC ACCCCTCAAG
CCTGCCAAGT CGGCC CGCTC CGTCCGTGCC CAGCGCCACA CGGACATGCC
CAAGGCTCAG AAG..(exon 5)...GAAGTACATT TGAAGAACAC AAGTAGAGGG
AGTTCAGGAA ACAAGAACTA CAGAATG

Sequence encoding the mature 70 amino acid IGF-I peptide is underlined. (Compiled from sequences published by Tavakkol et al., 1988; Müller & Brem, 1989; Weller et al., 1993).

1.3.3 Gene expression

In humans, transcription is initiated at one of the two leader exons, termed leader exon 1 or 2. Within each of the leader regions are multiple start sites for initiation of transcription. In the human, 80% of the leader exon 1 mRNA products (*class 1*) are initiated at a transcription start site 245 base pairs (bp) upstream of the leader exon 1-intron 1 junction, with several more minor transcription start sites upstream from this. In leader exon 2, *class 2* mRNA transcripts are generated from a heterogeneous population of transcription start sites ranging from 75 to 65 bp upstream of the leader exon 2-intron 2 junction, with several more

transcription start sites upstream from this position (Jansen *et al.*, 1991). In the rat, two major transcription start sites are found in leader exon 1 yielding *class 1* IGF-I mRNAs, while *class 2* mRNAs are generated from multiple clustered transcription start sites in leader exon 2 (Simmons *et al.*, 1993).

In the rat, IGF-I *class* 1 transcripts are found in all tissues, whereas IGF-I *class 2* transcripts are mainly found in the liver with low levels of expression in kidney, testes, lung and stomach. In all tissues, IGF-I *class 1* transcripts are at least 4 fold more abundant than IGF-I *class* 2 mRNA (Hoyt *et al.*, 1988; Adamo *et al.*, 1989; Shemer *et al.*, 1992).

In the pig, levels of IGF-I *class 1* transcripts appear to be similar in liver, longissimus dorsi, heart and soleus muscle, however, IGF-I *class 2* transcripts are 20-30 times lower in muscle than in liver (Weller *et al.*, 1993). Increasing the growth rate of pigs by manipulation of energy status increases plasma IGF-I levels and this is associated with increased levels of IGF-I *class 1* and *class* 2 mRNA in the liver. In addition, an increase in the ratio of hepatic IGF-I *class 2* to *class 1* mRNA is also observed with changes in energy status (Weller *et al.*, 1993; 1994).

In humans, two precursor forms of IGF-I are generated through differential splicing of its mRNA at the 3' end of the gene. IGF-I Ea is generated by splicing exon 4 to exon 6, while IGF-I Eb is comprised of exons 1 through to 5 (Figure 1.3). Since the sequence encoding the mature 70 amino acid IGF-I peptide (the B, C, A and D domains) is localised to exons 3 and 4, the two forms of precursor IGF-I differ in the carboxy-terminal, with IGF-I Ea comprising an additional 19 amino acids encoded by exon 6 and IGF-I Eb an additional 61 amino acids encoded by exon 5 (Jansen *et al.*, 1983; Rotwein 1986). As in the human, two possible IGF-I precursor proteins have also been identified in the rat (Lowe *et al.*, 1988). Postnatally, IGF-I Ea is expressed in a large range of tissues, suggesting that this prohormone is involved with local IGF-I action. IGF-I Eb is mainly found in the liver and is regulated by GH in hypophysectomized rats (Lowe *et al.*, 1988).

1.4 IGF-I Regulation and Action

IGFs regulate the differentiation and proliferation of a multitude of cell types and exert insulin-like effects by increasing glucose metabolism, stimulating glucose transport, glycolysis and increasing glycogen and protein synthesis (Sara and Hall, 1990). IGF-I is produced in most tissues in the body and is readily secreted into the circulation. Thus it has the potential to act in both an endocrine and auto/paracrine manner.

1.4.1 IGF-I action during embryonic and foetal development

IGF-I stimulates DNA synthesis in a variety of foetal cell types including dermal fibroblasts (Atkinson *et al.*, 1987), myoblasts from skeletal muscle (Florini *et al.*, 1977), chondrocytes (Vetter *et al.*, 1986), and hepatocytes (Strain *et al.*, 1987). In chondrocytes IGF-II is more potent than IGF-I at stimulating DNA synthesis. IGF-I also plays a role in foetal cellular differentiation. In chick embryo cartilage, IGF-I causes elongation of the glycosaminoglycan chains (Silbergeld *et al.*, 1981) while in chondrocytes from human foetal epiphyseal growth plate, IGF-I has differentiating actions by stimulating synthesis of the extracellular matrix (Hill 1979) as well as having proliferative actions. IGF-I has also been shown to have an effect on cellular differentiation in 1 day old rat oligodendrocytes (McMorris & Dubois-Dalq, 1988) and dorsal root ganglia (Xue *et al.*, 1988).

During human foetal development, IGF-I mRNAs are detected from 12 weeks of gestation in most tissues but at a much lower level than IGF-II (Han *et al.*, 1988). Foetal plasma IGF-I levels are also present from about 15 weeks of gestation (Ashton *et al.*, 1985), but are lower than those in adult or maternal sera (D'Ercole *et al.*, 1980). From about 32 weeks of gestation, concentrations of IGF-I and IGF-II increase but are still lower than in adult serum (Gluckman and Brinsmead, 1976). Although levels of IGF-II are much higher than IGF-I in the foetus, it is plasma and chord blood IGF-I levels that are correlated with the birthweight of the newborn (Hall *et al.*, 1986; Foley *et al.*, 1980).

Studies in the rodent foetus have shown a similar distribution of IGF expression. Both IGF-I and IGF-II mRNA are expressed as early as day 11 of gestation in a wide range of tissues (Rotwein *et al.*, 1987; Beck *et al.*, 1987) and IGF-II mRNAs are more abundant than IGF-I mRNAs in most foetal tissues (Moses *et al.*, 1980). This suggests that IGF-II may be the foetal growth factor that mediates growth and differentiation in foetal tissue, although the importance of IGF-I in foetal growth can not be discounted due to the strong correlation between foetal IGF-I levels and birth weight.

The importance of IGF-I and IGF-II in foetal development has been established by studies in transgenic mice with disrupted IGF-I or IGF-II genes. These transgenic mice are commonly refered to as "knockout transgenic mice". It has been shown that mice that are homozygous for the disruption of the IGF-I gene are growth retarded whereas mice that are heterozygous for the disrupted IGF-I gene show no or minimal growth retardation (Baker *et al.*, 1993; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). The majority of IGF-I knockout mice die immediately after birth, possibly due to respiratory distress, while those that do survive grow very slowly and are infertile (Baker *et al.*, 1993). IGF-II knockout mice also exhibit

growth retardation, but this is confined to only a short period during foetal life. For the remainder of the foetal period their growth rate is only minimally reduced (DeChiara *et al.*, 1990). These studies are compelling evidence for the importance of both IGF-I and IGF-II during foetal development and indicate that they have different and specific effects on prenatal growth.

1.4.2 IGF-I action on growth

In humans, IGF-I levels are low during the pre and neonatal periods, and begin to rise rapidly around the time of growth hormone dependence, reaching a peak during puberty and then declining with age (Hall and Sara, 1984). Pigs and sheep show a similar pattern of IGF-I expression, being low in the foetus, increasing during the second half of gestation and then increasing to a maximum level in the neonatal period (Lee *et al.*, 1990). Plasma IGF-I correlation with growth has also been observed in the rat (Unterman *et al.*, 1993) and IGF-I transgenic mice (Matthews *et al.*, 1988). In rats, plasma IGF-I levels are highest during the pubertal growth phase (Maes *et al.*, 1983) and hypophysectomy (Guler *et al.*, 1988). In humans, IGF-I levels are decreased in conditions associated with reduced growth rate such as Laron dwarfism (Laron *et al.*, 1971).

In contrast, there are no gestational changes in plasma IGF-I levels in foetal guinea pigs (Daughaday *et al.*, 1986). The growth rate of different strains of pigs also does not appear to be related to plasma IGF-I levels (Owens *et al.*, 1990). These examples appear to be an exception to the rule, where generally, plasma IGF-I levels are related to foetal, neonatal and postnatal growth.

1.4.3 Regulation of IGF-I expression by growth hormone

Growth hormone has been established as the major regulator of IGF-I gene expression. Elevation of plasma IGF-I levels by GH treatment is often associated with an increase in the number of high affinity GH receptors in the liver (Breier *et al.*, 1985). Plasma IGF-I levels have been shown to be significantly correlated with [^{125}I] -bGH binding to hepatic membranes as well as plasma GH binding protein (GHBP) levels in pigs injected with pGH (Ambler *et al.*, 1992). In rats, intermittent GH injection increases liver IGF-I protein and mRNA levels without altering liver GH receptor mRNA. In contrast, GH infusion increases the number of liver GH binding sites but this is associated with only a moderate increase in liver IGF-I

mRNA levels (Maiter *et al*., 1992). These results demonstrate that regulation of hepatic IGF-I expression by GH is not only regulated by the abundance of GH receptors.

GH regulates the expression of the IGF-I gene at the transcriptional level. In adult human liver, promoter 1 (P1) located upstream of leader exon 1 accounts for approximately 80% of IGF-I transcripts (Jansen *et al*., 1992) which are stimulated with GH treatment (Bichell *et al*., 1992). Transient transfection studies have shown that two liver-enriched transacting transcription factors, CCAAT/enhancer binding protein α (C/EBPα) and liver-enriched activating protein (LAP) increase the activity of P1. Their binding sites have been located to 119 base pairs upstream of the major transcription start site within exon 1 by DNase footpriniting (Nolten et al., 1994). These DNA-protein binding sites within P1 are not altered by GH treatment (Thomas *et al*., 1994), indicating that the transcriptional response to GH treatment is not mediated through these transcription factors. Recently, a similar situation has been found to exist within the promoter region of exon 2. Similarly, although transcription from promoter 2 is induced with GH treatment, DNase footprinting studies revealed that there was no difference between DNA-protein binding sites within promoter 2 between GH treatment and control (Le Stunff *et al*., 1995).

A single GH-regulated chromatin alteration within the IGF-I gene has been identified in transcripts originating from either promoter. This GH-inducible change has been mapped to the second intron of the IGF-I gene (Bichell *et al*., 1992). The role of this region in inducing transcription of the IGF-I gene in response to GH treatment remains to be defined.

A plethora of literature exists demonstrating the ability of GH to increase IGF-I mRNA and protein levels. Plasma IGF-I levels correlate with spontaneous GH secretion in healthy individuals (Blum *et al*., 1993a). The pubertal growth spurt in humans is associated with increased growth hormone production and elevated plasma IGF-I levels, and in adults, plasma IGF-I levels decline with age (Hall and Sara, 1984) due to a decrease in the production of growth hormone (Marcus *et al*., 1990). In medical conditions of elevated plasma GH levels such as acromegaly, patients exhibit elevated levels of plasma IGF-I (Hall and Sara, 1984).

In pigs, exogenous administration of GH increases circulating IGF-I levels with doses of at least 50 μg/kg liveweight being required to elicit a response (Chung *et al*., 1985). This is at least a 10 fold higher level of GH than is seen under normal physiological conditions. The role of GH in regulating foetal IGF-I expression is indicated by the decrease in plasma IGF-I levels after foetal hypophysectomy in pigs and sheep (Jewell *et al*., 1989; Mesiano *et al*., 1989). Plasma GH levels do not always correlate with plasma IGF-I levels during postnatal growth. In the pig, plasma GH levels increase significantly between 10 and 20 kg liveweight but show only a modest increase in plasma IGF-I, while in older animals (between 20 and 35

kg liveweight), plasma IGF-I concentrations are more closely correlated with increases in plasma GH (Owens *et al.*, 1991). These results demonstrate the complexity of IGF-I regulation during development, indicating that GH may not be the primary regulator of IGF-I during the earlier growth period.

The liver is suggested to be the major source of GH-regulated IGF-I in many mammalian species including rodents, humans, sheep and pigs. The levels of IGF-I mRNA in liver is far more than in other tissues. In the rat, the mRNAs for IGF-I in heart, skeletal muscle (Isgaard *et al.*, 1989), liver (Roberts *et al.*, 1986) and white adipose tissue (Peter *et al.*, 1993) are decreased following hypophysectomy and normalise with the administration of human GH. In the liver, a coordinate increase of all IGF-I mRNA splicing and polyadenylation variants is observed, indicating that in the rat, neither of these two RNA processing events are differently altered by GH (Bichell *et al.*, 1992). In the pig, regulation of IGF-I gene expression by GH has also been observed in preadipocytes (Gaskins *et al.*, 1990), liver, skeletal muscle (Grant *et al.,* 1990) and adipose tissue (Wolverton *et al.*, 1992).

IGF-I levels can be significantly altered by a combination of altered GH levels and nutrition. Plasma IGF-I concentrations are decreased by starvation, and raised by GH treatment in fed lambs (Hua *et al.*, 1993), indicating that the IGF-I-GH axis is uncoupled in catabolic states. Pell *et al.* (1993) showed that in sheep, IGF-I *class 1* and *class 2* transcripts are differentially regulated by nutrition and GH. Increases in liver IGF-I *class 2* transcripts correlate with an increase in the nutritional plane, while GH treatment causes a further increase. IGF-I *class 1* transcripts show a smaller and less sensitive response to GH, and only have an effect at the highest plane of nutrition. In the rat, IGF-I *class 1* transcripts increase in a linear fashion from birth to at least 50 days, while increases in IGF-I *class 2* transcripts do not occur until 20-30 days postnatally (Adamo *et al.*, 1989; 1991). This is consistent with the onset of GH responsiveness and suggests that exon 2 may be particularly sensitive to GH.

1.4.4 Nutritional regulation of IGF-I

Plasma IGF-I concentrations are significantly affected by energy status. Fasting, a low protein diet and a reduction in environmental temperature have been shown to decrease plasma IGF-I levels. Plasma IGF-I levels are significantly greater in young pigs acclimatised to a warmer environmental temperature, and in those animals on a higher energy intake (Dauncy *et al.*, 1990). Rats show a 60% decrease in plasma IGF-I after only 24 hours of fasting, which is corrected upon refeeding (Phillips and Young, 1976). This decrease in plasma IGF-I is associated with a reduction in the abundance of IGF-I and GH receptor mRNA in rat liver,

and skeletal muscle (VandeHaar *et al*, 1991) without affecting pituitary GH expression (Chan *et al.*, 1993). In obese man, a 25 % decline in plasma IGF-I is observed within 24 hours of fasting (Clemmons *et al.,* 1981). Nutritional deprivation has also been reported to reduce plasma IGF-I in dogs (de Bruijne *et al.*, 1985), cattle (Breier *et al.*, 1988), sheep (Bass *et al.*, 1991) guinea pigs (Palka *et al.*, 1989) and pigs (Buonomo and Baile, 1991). This decrease in plasma IGF-I is associated with a decrease in both the protein and mRNA levels in kidney but not muscle or liver of starved lambs (Hua *et al.*, 1993). The suppression of plasma IGF-I due to nutritional deprivation is not associated with a reduction in plasma GH concentration, in fact, plasma GH levels have been shown to increase, while plasma IGF-I levels are decreased. This may indicate that the GH-IGF-I axis is uncoupled during nutritional restriction. In addition, insulin levels are reduced during feed deprivation, while plasma glucose levels remain unchanged (Buonomo and Baile, 1991). Nutritional regulation of IGF-I appears to be independent of GH status. During feed restriction, plasma GH levels do not elevate in heifers that are actively immunised against growth hormone releasing factor (GRF), but plasma IGF-I and insulin levels are decreased in both GRF immunised and control groups (Armstrong *et al.*, 1993). Conversely, rams fed a high protein diet show a decrease in plasma GH, while plasma IGF-I levels are elevated. Pituitary GH mRNA is unaltered, while GH pituitary content is increased (Clarke *et al.,* 1993).

1.4.5 Regulation of IGF-I by other hormones and growth factors

Other growth factors can regulate IGF-I expression. Oestrogen treatment of ovariectomized rats increases IGF-I mRNA levels in the anterior pituitary (Michels *et al.*, 1993), suggesting that pituitary derived IGF-I may act as a mediator for oestrogen-induced effects of the anterior pituitary.

Glucocorticoids inhibit somatic growth and are well known for their ability to suppress GH secretion and IGF activity. Dexamethasone treatment of rats results in an increase in plasma IGF-I levels (Luo and Murphy, 1989). These results are supported by studies in man where dexamethasone administration produces a 30% increase in plasma IGF-I levels (Miell *et al.*, 1993a). This is associated with an increase in plasma insulin and IGFBP-3 levels, while IGFBP-1 and -2 concentrations are suppressed. Although *in vivo*, IGF-I transcription is decreased with dexamethasone treatment (Luo and Murphy, 1989), the rate of IGF-I clearance is altered, possibly due to the increased availability of IGFBP-3. In addition, glucocorticoids may alter the bioactivity of IGFs by the induction of inhibitors.

Thyroid hormones are important regulators of somatic growth in man and have also been shown to regulate IGF-I expression and bioactivity. Hypothyroidism in man reduces IGF-I levels and IGF bioactivity, while in hyperthyroidism, IGF-I levels are normal or high (Miell *et al.*, 1993b).

IGF-I expression is also regulated by a variety of other growth factors. Insulin has been shown to increase IGF-I gene transcription in primary rat hepatocytes (Pao *et al.*, 1993). Basic fibroblast growth factor (FGF) is stimulatory for IGF-I production in neuronal cells, glial cells (Pons and Torres-Aleman, 1992) and chondrocytes which also show increased production of IGF-I when treated with transforming growth factor (TGF)-β1 (Elford and Lambert, 1990). In human fibroblasts, platelet-derived growth factor (PDGF), FGF and epidermal growth factor (EGF) all induce the production of IGF-I (Clemmons 1984), while in pig granulosa cells only EGF and TGF-α are stimulatory (Mondschein and Hammond, 1988).

1.5 The Role of IGF-I in Tissue Growth and Differentiation

IGF-I has a wide range of physiological actions. It is generally considered to be a post-natal growth factor, since plasma levels of IGF-I are generally lower in the foetus in most species, and begin to rise after birth.

1.5.1 Metabolic actions of IGF-I

The growth promoting and insulin-like effects of IGF-I have been well documented (Sara and Hall, 1990), and its metabolic actions closely resemble those of insulin. IGF-I stimulates glucose oxidation, glycogen synthesis and amino acid transport (Baxter, 1988) and lowers creatinine, urea, triglyceride and cholesterol levels (Guler *et al.*, 1989). IGF-I increases total body weight gain and contributes to increasing selective organ growth such as the kidneys, spleen, thymus (Guler *et al.*, 1988), regenerating liver (Caro *et al.*, 1988), bone and cartilage (Daughaday and Rotwein 1989), heart and vasculature (Thomas *et al.*, 1993), gut (Read *et al.*, 1991) the nervous system (Carson *et al.*, 1993) and the reproductive organs (Murphy *et al.*, 1987). This list is by no means exhaustive.

1.5.2 Mitogenic actions of IGF-I

IGF-I is a progression factor and plays a vital role in cellular proliferation. It is a necessary component that allows cells to progress from the Gap1 (G_1) phase of the cell cycle to the S phase where cells prepare for DNA synthesis and chromosome replication (Pardee, 1989). The mitogenic effects of IGF-I have been demonstrated in many cell types by measuring DNA, RNA and protein synthesis. IGF-I has been shown to be mitogenic in cells such as fibroblasts and myoblasts (Hill *et al.*, 1986), chondrocytes (Vetter *et al.*, 1986), osteoblasts (Hock *et al.*, 1988), hepatocytes (Strain *et al.*, 1987) and glial cells (Han *et al.*, 1987). In other cell types, a synergism between IGF-I and other growth factors such as FGF, EGF or PDGF is required for stimulation of DNA synthesis.

1.5.3 Differentiative actions of IGF-I

IGF-I stimulates cellular differentiation of many different cell types. In myoblast cell lines established from neonatal rat muscle, IGF-I enhances the differentiation of myoblasts into contractile tubules (Ewton and Florini, 1981). During cartilage formation, IGF-I plays a critical role in the elongation of pre-existing glycosaminoglycan chains (Silbergeld *et al.*, 1981), while further cellular differentiation into mature cartilage requires the presence of other co-factors. Similarly, chondrocytes of the epiphyseal growth plate respond to IGF-I by stimulating the synthesis of the extracellular matrix (Hill, 1979). The role of IGF-I in brain maturation is implicated by its ability to initiate differentiation of oligodendrocytes to oligodendroglial formation (McMorris and Dubois-Dalq, 1988). IGF-I also exhibits differentiated functions in the reproductive organs. In granulosa cells, IGF-I acts in synergism with follicle-stimulating hormone to induce the receptors for luteinising hormone and stimulate steroidogenesis (Adashi *et al.*, 1985) while similar synergistic effects have also been seen in the testis (Lin *et al.*, 1986). In these cases of synergism, it appears that when IGF-I is expressed as a paracrine factor, it interacts with trophic endocrine hormones to initiate the differentiation event.

1.5.4 Anti-apoptopic actions of IGF-I

IGF-I has been shown to prevent the premature death of different cell types. Addition of IGF-I to the culture medium inhibits apoptosis in erythroid colony forming cells (Muta and Krantz, 1993) and both IGF-I and the IGF-I receptor are required for survival of cultured hematopoietic cells (Rodriguez-Tarduchy *et al.*, 1992). IGF-I is able to block cyclohexamide induced cell death (Geier *et al.*, 1993). In fibroblasts, IGF-I prevents apoptosis in cells exposed to topoisomerase inhibitor (Sell *et al.*, 1995), and both IGF-I and PDGF have been found to inhibit c-Myc induced apoptosis in serum deprived fibroblasts (Harrington *et al.*, 1994).

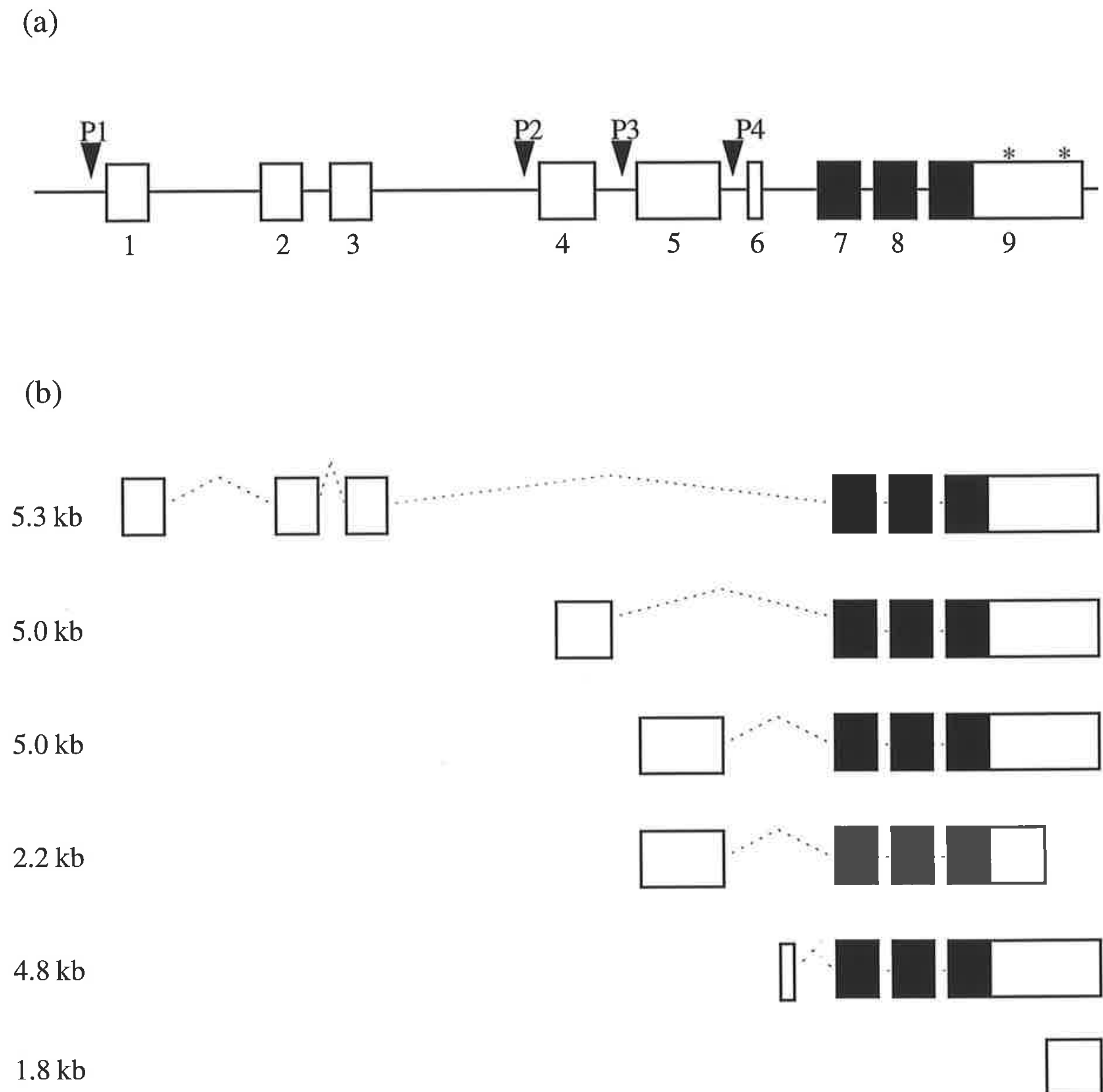
1.6 IGF-II Gene Structure and Expression

Like IGF-I, IGF-II is a single-chain polypeptide, but consists of 67 amino acids. IGF-II shares homology with pro-insulin through the existence of homologous B, C and A domains. As with IGF-I, IGF-II also has further carboxy-terminal extensions, the D domain and E domain, the latter of which is cleaved off in the mature peptide (Rinderknecht and Humbel, 1978b). IGF-II differs by one residue between humans and pigs, with up to six differences occurring with other species. As with IGF-I, the majority of the amino acid changes are located in the B and C domains.

The human IGF-II gene consists of 9 exons and resides on chromosome 11 spanning approximately 30 kb. Exons 1 to 6 are noncoding leader exons, with the precursor protein being encoded by exons 7, 8 and some of exon 9. Transcription is initiated from one of 4 promoters which are development and tissue specific. Further mRNA heterogeneity is introduced by differential transcription of the E domain sequence to produce varying lengths of the extension peptide (Rotwein, 1986) (Figure 1.5).

Figure 1.5:

The human IGF-II gene and its different mRNA species



The human IGF-II gene (a). The numbered boxes correspond to the different exons. P1 to P4 indicate the four different promoters. The six possible different mRNA species are shown (b). Solid boxes represent exon sequences encoding pre-pro-IGF-II and open boxes are exon sequences encoding untranslated mRNA. Splicing between alternatively used exons is indicated by the broken lines. (Adapted from Holthuizen et al., 1994).

1.7 IGF-II Action

IGF-II has an important role in regulating foetal development and mammalian growth. It is considered to be the foetal growth factor and its plasma concentration in foetal rats (Moses *et al.*, 1980), sheep (Gluckman and Butler, 1983) and guinea pigs (Daughaday *et al.*, 1986) is much higher than in the postnatal animal. In fact in rats, the levels of IGF-II in the adult animal are negligible (Donovan *et al.*, 1989). In contrast, IGF-II does not change postnatally in either humans (Engberg and Hall, 1984) or pigs (Owens *et al.*, 1991). Its vital role in foetal development was demonstrated by DeChiara *et al.* (1990) who showed that mice carrying a defective IGF-II gene exhibit considerable growth retardation. An elegant study by Liu *et al.* (1989) demonstrated that 10 day old rat embryos implanted into growing rats under the kidney's capsule grew in response to IGF-II but not IGF-I infusion into the host's renal artery.

IGF-II mRNA expression in the foetus has been most extensively studied in rodents. Expression in mice has been detected as early as the two-cell stage of the pre-implantation embryo (Rappolee *et al.*, 1989) and is abundant during mid-gestation where it has been identified in yolk sack, liver, lung and differentiating tissues of mesodermal origin (Ward and Elliss, 1992). During late foetal development and the neonatal period, IGF-II levels decline rapidly depending on the tissue. Similar trends in foetal IGF-II expression have been observed in humans (Brice *et al.*, 1989), cattle (Boulle *et al.*, 1993), pigs (Lee *et al.*, 1993b) and sheep (Delhanty and Han, 1993), although in these species IGF-II expression still persists in adult liver. Although IGF-I is considered to be the postnatal growth factor and IGF-II the foetal growth factor, it is important to note that IGF-I does affect foetal growth, and IGF-II is present in some adult tissue. Therefore this delinearisation of the role of the two IGFs in growth is not that clear cut.

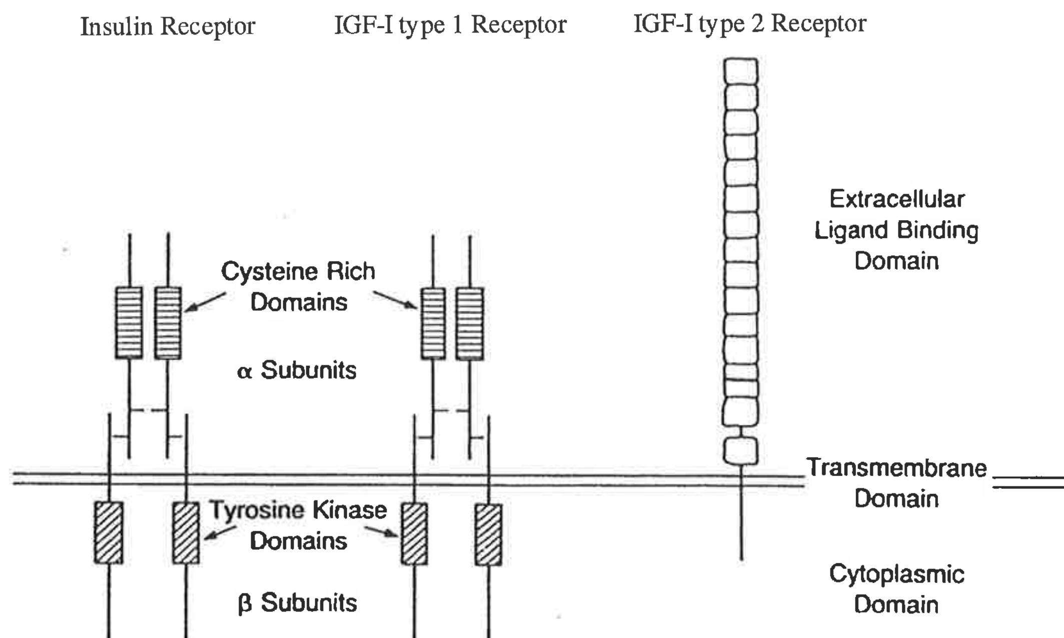
1.8 The IGF Receptors

The biological actions of the IGFs could in theory, be mediated by one of three receptors; the insulin receptor and the type I or type II IGF receptor. The majority of IGF mediated actions are via the type I IGF receptor. The insulin and type I IGF receptor exhibit considerable sequence and structural homology. They both consist of two alpha and beta subunits linked through disulphide bridges, and have an intrinsic tyrosine kinase activity in the

β subunit (Figure 1.6). The tyrosine kinase activity is activated by ligand binding to the α subunit (Ullrich *et al.*, 1986). The insulin receptor has a high affinity for insulin and binds the IGFs only weakly, whereas the type I IGF receptor binds both IGFs strongly and has only a weak affinity for insulin (reviewed by Sara and Hall, 1990). The type 2 IGF receptor is structurally unrelated in that it consists of a single polypeptide chain that does not exhibit any tyrosine kinase activity (Corvera *et al.*, 1986). Only IGF-II binds to this receptor with high affinity. Its main physiological role is the targeting of mannose-6-phosphate containing enzymes to lysosomes.

Figure 1. 6:

Schematic representation of the IGF and insulin receptors



(Adapted from LeRoith et al., 1992)

1.8.1 The type-1 IGF receptor

IGF-I and IGF-II both mediate their effects through the type 1 IGF receptor which is widely distributed throughout the different organs of the body. The type 1 I IGF receptor has been localised to human chromosome 15 (Ullrich *et al.*, 1986) and is translated into two mRNA species. It is a heterotetrameric glycoprotein, consisting of two extracellular α subunits and two intracellular β subunits. The subunits are linked to their homologous subunit and the other subunits via disulphide bonds. The structure of the type 1 IGF receptor was first

recognised through ^{125}I-IGF-I affinity crosslinking (Chernausek *et al.*, 1981) and photoaffinity labelling techniques (Bhaumik *et al.*, 1981). Cells or membranes exhibiting high affinity IGF-I binding were shown to possess a specifically labelled 350 kDa protein, which was able to be reduced to a strongly labelled 135 kDa peptide (the α subunit) and a weakly labelled 90 kDa peptide (the β subunit) with dithiothreitol.

The sequence and organisation of the type 1 receptor exhibits strong homology with insulin. It is composed of a N-terminal signal peptide which is cleaved in the mature protein, followed by the α and β subunits. Within the α subunit between residues 223 and 274 is a cysteine-rich region, which is also present in the α subunit of insulin (see Figure 1.6). This region has been identified as being crucial for hormone binding specificity (Gustafson and Rutter, 1990). The β subunit exhibits regions characteristic for a transmembrane domain and a tyrosine kinase domain. These regions are also identified in the insulin receptor. The transmembrane domain is a series of hydrophobic amino acids followed by several basic residues which is characteristic of a cytoplasmic anchor. This cytoplasmic region contains the tyrosine kinase domain and the sites of autophosphorylation. Sequence homology 3' to this region diverges considerably between the type 1 and insulin receptors and is thought to be responsible for the differences in biological activity between the two receptors (Moxham and Jacobs, 1992).

Despite the extensive homology between the insulin and type 1 receptors, they have different physiological roles. The type 1 receptor is primarily involved with regulating growth and development using IGF peptides as the ligand, while the insulin receptor modulates the effects of insulin on metabolism. Nevertheless, IGF-I has been shown to have insulin-like effects in several cell types by acting through its receptor to stimulate glucose transport and glycogen synthase (Shimizu *et al.*, 1986; Schwartz *et al.*, 1985; Verspohl *et al.*, 1984; Steele-Perkins *et al.*, 1988). The type 1 receptor appears to be quantitatively more efficient than the insulin receptor in mediating mitogenic responses. IGF-I is more efficient than insulin in stimulating thymidine incorporation in rat cells transfected with human insulin receptors and expressing a similar level of type 1 receptors (McClain *et al.*, 1988).

IGF-I stimulates the tyrosine phosphorylation of its receptor and other proteins in intact cells and solubilised receptor preparations (Zick *et al.*, 1984; Shemer *et al.*, 1987). There is a strong consensus in the literature that the autophosphorylation of the β subunit leads to activation of the tyrosine kinase of the type 1 receptor and is necessary for exerting biological action (Moxham and Jacobs, 1992), however, the signal pathway that is followed in this event still remains to be clarified. The type 1 receptor also contains several

phosphorylated serines, probably catalysed by protein kinase C. This stimulates the intracellular cycling of the receptor and decreases autophosphorylation in response to IGF-I, possibly acting as a regulatory mechanism of IGF-I action (Jacobs and Cuatrecasas, 1986).

1.8.2 The type-2 IGF receptor

The cDNA sequence of the type 2 IGF receptor was cloned and sequenced by Morgan *et al.* (1987) and MacDonald *et al.* (1988). The 250 kDa protein is identical to the cation-independent-mannose-6-phosphate receptor (Kiess et al., 1988) and is composed of a large N terminal extracellular domain, a single transmembrane region and a small cytoplasmic tail. The extracellular domain is composed of 15 repeat sequences containing a pattern of 8 conserved cysteine residues that are structurally unrelated to those found in the type 1 or insulin receptor, and the cytoplasmic tail can be cleaved to produce a circulating form of the type 2 receptor (Kiess *et al.*, 1987). The extracellular domain binds IGF-II and mannose-6-phosphate at separate sites, and binding of one ligand does not inhibit binding of the other (Morgan *et al.*, 1987). IGF-II does not bind to the mannose 6-phosphate receptor in chicken or *Xenopus* (Clairmont & Czech, 1989; Yang *et al.*, 1991) and binds to the opossum homologue with a lower affinity than to the bovine receptor (Dahms *et al.*, 1993).

The most likely role of the type 2 IGF receptor is to bind and internalise IGF-II. The majority of type 2 receptors are located intracellularly in low-density microsomal membranes (Wardzala et al., 1984). The number of cell surface receptors can be regulated by redirecting intracellular receptors to the cell surface (Braulke *et al*, 1990). Treatment of cells with insulin results in a rapid increase in IGF-II binding to intact cells caused by a redistribution of the intracellular receptor to the cell surface (Wardzala et al., 1984). The rate of IGF-II internalisation is independent of IGF-II binding to the receptor, but rather depends on the rate at which the type 2 receptor shuttles between the cell membrane, the trans-Golgi network and the prelysosomal compartment (Duncan and Kornfeld, 1988). The main function of the type 2 receptor is to transport lysosomal enzymes with mannose-6-phosphate attachments from their site of synthesis to the acidic prelysosomal compartments (Braulke *et al.*, 1990).

There is a limited amount of evidence to suggest that IGF-II mediates some of its actions via this receptor. IGF-II has been shown to stimulate the influx of Ca^{2+} ions into cells (Nishimoto *et al*, 1987), stimulate production of inositol triphosphate (Rogers and Hammerman, 1988) and diacylglycerol (Rogers *et al.*, 1990) and increase the motility of

rhabdomysarcoma cells (Minniti *et al.*, 1992). A direct biological consequence of activation of this receptor has yet to be clearly demonstrated.

1.9 The IGF Binding Proteins

In the circulation, the IGFs are bound to the insulin-like growth factor binding proteins (IGFBPs). Their role is to transport the IGFs in the circulation and across the capillary membranes, increase the circulating half life of the IGFs, localise them to specific tissue and cell types (Ballard *et al.*, 1991) and regulate the interactions between IGFs and their receptors. In this capacity they act to either potentiate or inhibit IGF action.

To 1993, 6 IGFBPs have been identified and are known as IGFBP-1, -2, -3, -4, -5 and -6. They share high structural homology, but have unique characteristics and show development and tissue specific expression (Rechler, 1993). All 6 binding proteins contain at least 18 cysteine residues that are aligned at the amino and carboxyl termini, and a cysteine free region in the central area of the protein that shows very little sequence homology (Clemmons, 1993). Post-translational modification of the binding proteins by glycosylation, phosphorylation and proteolysis alter their ability to bind IGFs and thus affect their potency in modulating IGF action.

Usually, IGFBPs are inhibitory to IGF action by preventing their binding to the receptors, however in some cases they may also stimulate the actions of IGFs (Elgin *et al.*, 1987, Blum *et al.*, 1989). The proposed model of IGFBP action is that they act as blocking agents, preventing IGF binding to the cell-surface receptor. IGFBP-1 and IGFBP-3 have been shown to block ^{125}I-IGF-I association with cell surface receptors in several cell types (Pekonen *et al.*, 1988, Ritvos *et al.*, 1988, Walton *et al.*, 1989a), and the potency of this blocking effect is directly related to their affinity to bind IGFs. The cell surface bound IGFBPs may act as a reservoir of binding sites for IGFs, effectively competing with the IGF receptors (DeVroede *et al.*, 1986). In addition, free IGFBPs also compete for IGF binding, and have a much higher affinity for IGFs than do membrane bound IGFBPs (McCusker *et al.*, 1990).

1.9.1 IGFBP-1

1.9.1.1 IGFBP-1 structure

IGFBP-1 is identical to placental protein 12 (Chard, 1989), a protein originally isolated from human placenta, and has been shown to have similar biochemical and immunological properties as pregnancy associated endometrial α_1 globulin, chorionic α_1 -microglobulin, placental-specific α_1 -microglobulin and endometrial protein 14 (Drop *et al.*, 1991). IGFBP-1 is a non-glycosylated protein with a molecular mass of 25 kDa, but appears at approximately 28 kDa on western ligand blots (Rechler, 1993). It has a carboxy-terminal receptor recognition (RGD) sequence, implicating its binding to the cell surface (Rechler, 1993). IGFBP-1 exists in both a phosphorylated and non-phosphorylated form. When phosphorylated, IGFBP-1 inhibits IGF-I action as measured by DNA synthesis, whereas the non-phosphorylated form is stimulatory to IGF-I action (Clemmons, 1993). IGFBP-1 is readily transported across intact endothelium and capillaries (Bar *et al.*, 1990), and has equal affinities for IGF-I and -II (Baxter *et al.*, 1987). Insulin stimulates the transport of IGFBP-1 across the endothelium (Bar *et al.*, 1990). This also occurs when IGF-I is bound to IGFBP-1.

1.9.1.2 IGFBP-1 expression

In humans, IGFBP-1 levels are high in amniotic fluid, cord serum and retroplacental serum (Drop *et al.*, 1984). In contrast, no IGFBP-1 activity can be found in the amniotic fluid of the pig (Veomett *et al.*, 1989). It is the primary circulating binding protein in foetal and maternal human sera and reaches peak values in the second trimester, decreasing thereafter (Baxter *et al.*, 1987). A strong correlation between cord serum IGF-I : IGFBP-1 ratio and birth weight have been recorded in humans (Hall *et al.*, 1986) suggesting that this binding protein plays a crucial role in regulating the effect of IGF-I on the growth and development of the foetus. A similar ratio of plasma IGF-I : IGFBP-1 and birth weight has also been shown to occur in the pig (McCusker *et al.*, 1988). After birth, IGFBP-1 levels decrease and continue to decline throughout puberty, and its levels during this time are inversely related to IGF-I concentrations. After puberty, IGFBP-1 concentrations begin to increase again, while IGF-I concentrations decrease with age (Hall *et al.*, 1988). The decline in pubertal IGFBP-1 levels is associated with increased insulin secretion accompanying the onset of GH induced insulin

resistance. IGFBP-1 levels are also elevated in conditions where growth and metabolic rates are altered (Dunger *et al.*, 1991).

Predominant expression of IGFBP-1 occurs in liver, with the foetal tissue showing higher levels of expression (Rechler, 1993). In postnatal life, IGFBP-1 expression is limited to the liver, ovarian granulosa cells and secretory or decidualised endometrium (Brinkman *et al.*, 1988; Julkunen *et al.*, 1988; Suikkari *et al.*, 1989).

IGFBP-1 is the only binding protein that exhibits a diurnal pattern of expression with the lowest levels during the afternoon, followed by a several fold increase during the night. These night peaks disappear with regular night meals (Baxter and Cowell, 1987). During fasting, IGFBP-1 levels increase but the diurnal rhythm is maintained despite a suppression of insulin secretion (Brismar & Hall 1993). Similarly, in cases of GH deficiency, IGFBP-1 concentrations are elevated, but the circadian rhythm and the inverse relationship to insulin are maintained (Hilding *et al.*, 1993). In obese humans, the diurnal pattern is reversed, with depressed night peaks which can be attributed to increased insulin concentrations. Similar patterns of expression also exists in cases of acromegaly, which may be attributed to cortisol induced hyperinsulinaemia (Brismar *et al.*, 1991).

1.9.1.3 IGFBP-1 action

IGFBP-1 is proposed to be involved in glucose homeostasis (Lewitt *et al.*, 1993). Insulin inhibits both glucose and IGFBP-1 secretion from the liver (Lee *et al*, 1993a; Lewitt *et al.*, 1992), whereas glucagon stimulates gluconeogenesis and the expression of IGFBP-1 in foetal liver (Lewitt and Baxter, 1990). Hyperglycaemia stimulates insulin release which results in glucose uptake by tissues and the transport of the IGF/IGFBP-1 complex to the tissues (Bar *et al.*, 1990). High glucose levels have also been shown to inhibit IGFBP-1 production (Brismar and Hall, 1993). Conversely, hypoglycaemia induced by fasting, inhibits insulin release and elevates plasma IGFBP-1 levels (Brismar and Hall, 1993). This increase in IGFBP-1 may be to ensure that the brain in the absence of insulin can obtain enough glucose, since the elevated plasma IGFBP-1 levels can bind IGFs to inhibit their insulin-like effects in inducing glucose uptake by tissues. Obese patients have decreased levels of IGFBP-1 and a decreased insulin sensitivity. This allows for a greater fraction of available IGFs which increases tissue availability to stimulate glucose uptake. This concept is also supported during puberty and acromegaly, where IGF-I levels are high and IGFBP-1 levels are low, leading to a greater pool of available IGF-I, while during conditions of catabolism or malnutrition, IGF-I levels are low

and IGFBP-1 levels are elevated, ensuring that the majority of IGF-I is bound to IGFBP-1 (Hall *et al.*, 1988).

1.9.1.4 Modification of IGFBP-1

Post-translational modification such as phosphorylation and proteolysis alter the affinity of IGFBP-1 for IGFs. IGFBP-1 is phosphorylated at serine residues 101,119 and 169, with residues 101 being the most frequently phosphorylated. The pattern of phosphorylation is specific, as serines 99 and 98 are not phosphorylated even though they are favourable phosphorylation sites. Replacement of serine 101 with an alanine, results in a threefold reduction in the affinity for IGF-I, while dephosphorylation of the remaining two serine residues results in a further two fold reduction in affinity for IGF-I (Jones *et al.*, 1993). This suggests that serine 119 and 169 play an important role in IGFBP-1/IGF-I interactions. The potency of dephosphorylated IGFBP-1 was measured by assessing its effectiveness in wound healing (Jyung *et al.*, 1994). It was shown that dephosphorylated IGFBP-1 administered together with IGF-I results in a 31% increase in wound breaking strength when compared to phosphorylated IGFBP-1. Modulation of IGFBP-1 bioactivity through proteolitic cleavage has been shown to occur in amniotic fluid (Binoux *et al.*, 1994).

1.9.2 IGFBP-2

1.9.2.1 IGFBP-2 structure

IGFBP-2 is one of the smaller binding proteins with a molecular mass of 31.3 kDa (Blum *et al.*, 1993b). Like IGFBP-1, it easily crosses the endothelial barrier indicating that it may be important for IGF transport and contains a RGD sequence that facilitates binding to the integrin receptor on the cell surface (Bar *et al.*, 1990). IGFBP-2 has a greater affinity for IGF-II than IGF-I.

1.9.2.2 IGFBP-2 expression

IGFBP-2 shows developmental patterns of expression similar to IGF-II (Straus *et al.*, 1991). In the foetal rat, IGFBP-2 expression is found in lung, liver, kidney, brain, heart, muscle and stomach, with the highest levels detected in brain, which is also higher in foetal than postnatal tissues (Ooi *et al.*, 1990; Orlowski *et al.*, 1990). In adult rats expression is found in brain, kidney, liver and endocrine organs such as adrenals, ovaries and testes (Rechler *et al.*, 1991). This developmental switch of IGFBP-2 expression has also been observed in pigs (Kampman *et al.*, 1993) and humans (Blum *et al.*, 1993b; Schwander and Mary, 1993). High levels of this binding protein have also been found in the cerebrospinal fluid of rats and humans (Lamson *et al.*, 1989; Romanus *et al.*, 1989). Plasma IGFBP-2 levels are significantly higher in umbilical cord serum than in maternal blood, and show an age dependent decrease from birth to about 4 - 6 years. In human milk, IGFBP-2 levels are high while plasma levels in lactating mothers are within the normal range (Schwander and Mary, 1993).

1.9.2.3 IGFBP-2 action

The role of GH in regulating IGFBP-2 is still unresolved. Decapitation of foetal pigs decreases plasma IGFBP-2 levels (McCusker *at al.*, 1989) and neonatal hypophysectomy in mice produces a similar response (Glasscock *et al.*, 1990). However in adult pigs (McCusker *et al.*, 1990), rats (Orlowski *et al.*, 1990) and mice (Glasscock *et al.*, 1990) hypophysectomy results in elevated plasma IGFBP-2 levels. In addition, treatment of intact pigs with GH has shown a dose-dependent decrease of a 34 kDa binding protein, probably IGFBP-2 (Coleman and Etherton, 1991). The difference seen in IGFBP-2 response to GH treatment may be due to a difference in IGFBP-2 regulation in the foetus and neonate compared to the adult. In the adult it seems that GH has a direct suppressive effect on plasma IGFBP-2 levels. Patients with GH deficiency show a slight increase in IGFBP-2 levels, which is decreased during GH treatment. In contrast, patients with acromegaly show diminished plasma levels of IGFBP-2 which are restored after removal of the GH producing tumour (Blum *et al.*, 1993b).

IGF-I treatment increases the expression of IGFBP-2 in normal rats (Tomas *et al.*, 1993) and plasma levels of IGFBP-2 that have been suppressed by diabetes or nitrogen restriction can be restored with IGF-I treatment (Tomas *et al.*, 1991a, b). IGF-I administration to normal humans also produces an elevation in plasma IGFBP-2 levels (Baxter *et al.*, 1993).

Fasting produces different responses on IGFBP-2 expression depending on the age and species. In neonatal pigs (McCusker *et al.*, 1989), fasting causes a decrease in IGFBP-2, while in rats the plasma levels are elevated (Orlowski *et al.*, 1990). This is associated with an increase in hepatic IGFBP-2 mRNA production (Tseng *et al.*, 1992). In adults, a 3 day fast results in increased plasma IGFBP-2 levels which are normalised within one day of food intake (Blum *et al.*, 1993b).

Insulin also has different effects on IGFBP-2 expression depending on the species. Rats with streptozotocin induced diabetes have increased expression of hepatic IGFBP-2 mRNA which is reversible by insulin, and treatment of rat hepatocytes with insulin suppresses IGFBP-2 mRNA and protein expression (Schwander *et al.*, 1994). In humans there has been no evidence that such a relationship exists and there is no consensus hypothesis to explain the relationship between insulin and IGFBP-2 levels. IGFBP-2 levels have been shown to be depressed in situations where insulin levels are high (such as acromegaly) or low (such as insulin dependent diabetes). Increased levels of IGFBP-2 have also been reported in conditions where insulin levels are low (such as fasting and growth hormone deficiency) (Blum *et al.*, 1993b). Clemmons *et al.* (1991) have shown that insulin administration in the presence of a glucose clamp has no effect on plasma IGFBP-2 levels. These observations indicate that insulin is not a primary regulator of IGFBP-2 and that other regulatory factors override the effects of insulin in regulating IGFBP-2 levels.

1.9.3 IGFBP-3

1.9.3.1 IGFBP-3 structure

The human IGFBP-3 gene is located on chromosome 7 and spans a region of 8.9 kb. The protein sequence is encoded in 4 exons with a 5th exon containing the 3' untranslated region. Its organisation is relatively uncomplicated in that it exists as a single copy gene that is transcribed from one promoter to yield one mRNA species (Cubbage *et al.*, 1990). The porcine IGFBP-3 gene is 2 amino acids longer at 266 amino acids than its human counterpart. There are 42 amino acid substitutions between the two species, but the 18 cysteines and 3 Asn-linked glycosylation sites are conserved. Transcription of the porcine IGFBP-3 gene yields a single mRNA species 2.6 kb in length and has been detected in gonadal tissue, brain, liver, muscle, lung and kidney (Shimasaki *et al.*, 1990; Lee *et al.*, 1993b).

IGFBP-3 is the primary carrier of IGFs in the circulation, and has a greater affinity for IGF-I than IGF-II (Baxter and Cowell, 1987). This protein has been isolated and characterised in humans (Baxter and Martin, 1986), rats (Baxter and Martin, 1987) and domestic species such as cow (Conover *et al.*, 1990) and pig (Shimasaki *et al.*, 1990). In human plasma it appears as two glycoprotein forms 41.5 and 38 kDa in size, which appear as a single band of 31 kDa upon deglycosylation (Liu *et al.*, 1990). This is consistent with the predicted sizes obtained from sequence analysis of human, porcine, rat and bovine IGFBP-3 cDNA clones. Unlike some of the other IGFBPs, IGFBP-3 does not have a RGD binding sequence (Rechler, 1993). It has been suggested that the high degree of glycosylation of IGFBP-3 when compared to IGFBP-1 or -2 may account for some specific membrane interactions (Conover *et al.*, 1990).

1.9.3.2 IGFBP-3 expression

There is relatively little information on the tissue specific expression of IGFBP-3 in humans and pigs. IGFBP-3 expression has been most extensively studied in the rat. In this species, a single 2.6 kb band corresponding to IGFBP-3 has been detected in liver, kidney, stomach, heart, adrenal, ovary, testis, spleen, lung, small and large intestine, but no IGFBP-3 mRNA could be detected in hypothalamus or cerebral cortex of the adult rat (Shimasaki *et al.*, 1989). This wide distribution of IGFBP-3 suggests that it plays an important role in the autocrine/paracrine action of IGF-I although no correlation has been observed between IGF-I and IGFBP-3 mRNA expression in different tissues (Albiston and Herington, 1992). The major source of IGFBP-3 mRNA are kidney and liver (Albiston and Herington, 1992).

In pigs, rats and humans, low levels of plasma IGFBP-3 are detected during foetal development and these rise significantly during the postnatal period (McCusker *et al.*, 1989; Lee *et al.*, 1993b; Glasscock *et al.*, 1990) reaching peak values during puberty (Baxter and Martin, 1986; Blum *et al.*, 1990). This change in expression is co-ordinated with a decrease in IGFBP-2 levels (Donovan *et al.*, 1989). Plasma IGF-I and IGFBP-3 levels are closely related during the growth phase in humans (Argente *et al.*, 1993) rats (Donovan *et al.*, 1989; Glasscock *et al.*, 1990) and pigs (Lee *et al.*, 1993b).

Whether GH or IGF-I are the primary regulators of IGFBP-3 is still under contention. GH treatment increases plasma IGFBP-3 levels in rats (Clemmons *et al.*, 1989), humans (Laron, 1993) and pigs (Walton and Etherton, 1989). Increased plasma IGFBP-3 levels in response to GH treatment are also associated with an increase in plasma IGF-I. In

hypophysectomized rats, liver IGF-I and IGFBP-3 mRNA expression is reduced by 90% and 50% respectively and both are restored with GH treatment (Albiston and Herington, 1992). This would suggest that the effect on IGFBP-3 expression can not be attributed to GH alone since IGF-I levels are also depleted and then restoredd by GH treatment.

Patients with Laron syndrome typically have low levels of IGFBP-3 and IGF-I which do not change with GH treatment due to a defective GH receptor (Laron, 1993). Treatment with IGF-I results in normalisation of plasma IGF-I levels without significantly increasing plasma IGFBP-3 levels (Fielder *et al.*, 1993). In contrast, transgenic mice that express IGF-I in the absence of GH (IGF-I transgenic mice crossed to GH deficient dwarf mice), exhibit increased plasma IGFBP-3 levels, suggesting that IGF-I can regulate the expression of IGFBP-3 in the absence of GH (Camacho-Hubner *et al.*, 1991). In bovine fibroblasts, IGF-I treatment increases the expression of IGFBP-3 mRNA and protein. This is blocked when RNA synthesis is inhibited by actinomycin D. GH has no effect on IGFBP-3 mRNA or protein levels, and different analogues of IGF-I show differing levels of induction that are directly related to their ability to bind to the type 1 IGF receptor (Bale and Conover, 1992). In contrast, IGF-I treatment of human fibroblasts produces an increase in the level of IGFBP-3 protein without affecting the level of transcription (Bale and Conover, 1992). In this case it appears that IGF-I is inhibiting the degradation of IGFBP-3 rather than inducing its *de novo* synthesis.

The effects of IGF-I treatment on plasma IGFBP-3 concentrations vary depending on the species. In normal rats IGF-I treatment increases plasma levels of IGFBP-3 (Tomas *et al.*, 1993). In humans, treatment with IGF-I decreases plasma IGFBP-3 levels with no change in the urinary excretion of GH although the pulsatile activity of plasma GH levels was not examined (Baxter *et al.*, 1993). IGFBP-3 concentrations are lower in diabetes (Baxter and Martin, 1986) and hypothyroidism (Nanto-Salonen *et al.*, 1993). IGFBP-3 levels are depressed by fasting (McCusker *et al.*, 1989) and restricted protein feed intake (Zapf *et al.*, 1989). Insulin stimulates the expression of IGFBP-3 in ovarian granulosa cells (Grimes and Hammond, 1992) but with lower potency than IGF-I.

The majority of IGFBP-3 circulates as part of a large 150 kDa complex that is composed of IGF-I, IGFBP-3 and an acid-labile subunit (ALS) (Baxter and Martin, 1989). There is a close correlation between plasma IGFBP-3 and ALS levels and depletion of ALS may increase clearance of IGFBP-3. Infusing hypophysectomized rats with IGF-I elevates the levels of IGFBP-3 but not the formation of the 150 kDa complex, which is stimulated by GH infusion. It appears that plasma IGFBP-3 levels are controlled by growth hormone via the secretion of IGF-I, while the ALS is regulated directly by GH. (Zapf *et al.*, 1989).

1.9.3.3 Modification of IGFBP-3

Changes in circulating levels of IGFBP-3 during development and after hypophysectomy do not appear to be related to IGFBP-3 mRNA expression. Therefore post transcriptional regulation of IGFBP-3 may play an important role in the circulating levels of this protein. Limited proteolysis of IGFBPs by serine proteases alters the structure of some of the binding proteins and their affinity for IGFs. Some proteolysis of IGFBP-3 occurs in normal serum, but in serum of pregnant women, almost all of IGFBP-3 is degraded by proteolysis (Lamson *et al.*, 1991, Gargosky *et al.*, 1992). Increased protease activity has also been reported in catabolic states including a post-operative period after major surgery (Cwyfan Hughes *et al.*, 1992), severe illness (Davies *et al.*, 1991), in GH resistant and deficient states (Fielder *et al.*, 1992; Holly *et al.*, 1993), noninsulin-dependent diabetes mellitus (Bang et al., 1994) and malignancies (Muller *et al.*, 1993).

A relationship between GH status and the degree of IGFBP-3 proteolysis also exists. Up Generally up to about 35 % of IGFBP-3 produced by the liver is proteolysed, while GH deficient individuals show as much as 50%, whereas acromegalic patients exhibit only about 15% IGFBP-3 proteolysis (Lassarre *et al.*, 1994). This suggests that regulation of protease activity may be dependent on circulating levels of IGF-I. When IGF-I levels are low (and GH is low), protease activity is increased to increase the bioavailability of IGF-I, whereas bioavailability is decreased when circulating IGF-I levels are high. In Laron-type GH-insensitivity, intact IGFBP-3 levels are low, while the presence of proteolysed IGFBP-3 fragments is high. During IGF-I treatment of these individuals, the intact form of IGFBP-3 reappears, while the proteolytic fragment remains unchanged or decreases marginally (Binoux *et al.*, 1994).

1.9.4 IGFBP-4

IGFBP-4 is a N-glycosylated protein which was first identified in the medium of human osteosarcoma cells as a 25 kDa protein (Mohan *et al.*, 1989). Since then its mass has been predicted to be close to 26 kDa, and appears as a 24 kDa or 29 kDa band on western ligand blots depending on its state of glycosylation (Rechler, 1993).

IGFBP-4 mRNA is expressed in many human foetal tissues. These tissues also contain high levels of the type 1 and membrane bound type 2 IGF receptors, suggesting that IGFBP-4 play an important role in foetal growth and development (Delhanty *et al.*, 1993).

IGFBP-4 has been speculated to be an important negative regulator of bone formation. Agents that increase osteoblastic cell proliferation such as progesterone and the IGFs, inhibit the production of IGFBP-4, while those substances that inhibit cellular proliferation increase IGFBP-4 levels. This regulation occurs at the level of synthesis and degradation (Mohan *et al.*, 1994).

Protein levels of IGFBP-4 are decreased without affecting mRNA levels when human fibroblasts are treated with IGFs (Conover *et al.*, 1994), indicating that IGFBP-4 bioactivity is regulated by post-translational modification. Proteolytic cleavage of IGFBP-4 results in stimulation of IGF-I action. Addition of IGFBP-4 to fibroblasts that do not produce protease results in a dose dependent decrease in IGF-I stimulated DNA synthesis. However, addition of IGFBP-4 to smooth muscle cells does not inhibit IGF-I induced DNA synthesis. Analysis of the conditioned media shows that the ability for IGF-I to induce DNA synthesis is dependent on IGFBP-4 proteolysis (Clemmons, 1994).

1.9.5 IGFBP-5

At the time of completion of this literature review, IGFBP-5 had only recently been discovered and has not yet been extensively studied. It has a molecular mass of approximately 28 kDa (Rechler, 1993).

IGFBP-5 is O-glycosylated. It has many glycosaminoglycan sequences, allowing strong interactions with the extracellular matrix (Hodgkinson *et al.*, 1994). It is involved in regulating bone formation by increasing IGF actions on bone cells and incorporating IGF-II into the bone matrix. IGFBP-5 binds strongly to hydroxyapatite (Rechler, 1993). In contrast to IGFBP-4, IGFBP-5 production is stimulated by progesterone and IGFs (Mohan *et al.*, 1994). IGFBP-4 and -5 have been shown to be the only binding proteins present in granulosa cells of atretic follicles. To this extent, they have been suggested to be crucial for switching follicle progression towards growth or atresia (Shimasaki et al., 1994).

Like IGFBP-4, IGFBP-5 can also be cleaved by a serine protease. It is found in a proteolysed state in the condition media of osteoblast cell lines (Andress *et al.*, 1991). IGFBP-5 degradation is inhibited by IGF-I and especially IGF-II but not insulin, possibly due to the growth factor/binding protein complex masking the protease cleavage site (Clemmons, 1993).

1.9.6 IGFBP-6

IGFBP-6 has a molecular mass of approximately 22 kDa (Rechler, 1993). It is present in serum and cerebrospinal fluid and is expressed in epithelial and mesenchymal cells (Han et al., 1994). IGFBP-6 has a greater binding affinity for IGF-II than for IGF-I. Although IGFBP-6 contains a potential N-glycosylation site, it is not N-glycosylated but is O-glycosylated and removal of the O-linked oligosaccharides does not affect its binding to IGF-II (Bach *et al.*, 1993).

1.10 The Acid Labile Subunit

The majority of IGF-I and II in the circulation is bound to IGFBP-3. Chromatographic studies have shown that IGF-I and II migrate in two discrete complexes, one at approximately 50 kDa and a larger complex at 150 kDa (Barreca and Minuto, 1989). An additional peak at 210 kDa has also been detected which contains the circulating form of the type 2 receptor (Kiess *et al.*, 1987). The 150 kDa complex is the principal carrier of IGFs in the circulation (Borsi *et al.*, 1982) and is decreased in patients with growth hormone deficiency, diabetes and during fasting (Arner *et al.*, 1989). It is composed of three subunits, IGF-I or II, IGFBP-3 and an acid-labile subunit (Baxter *et al.*, 1989). Formation of this complex requires the binding of IGF-I or II to IGFBP-3 before the ternary complex can be formed. Upon exposure to acid, the complex dissociates into IGF, IGFBP-3 and the acid labile subunit whose binding activity is destroyed by acid treatment, hence its name. The ternary complex regulates IGF action by increasing the half life of the IGFs. This is evident in that that the 150 kDa complex is cleared more slowly than either the 50 kDa complex or free IGFs. The acid labile subunit blocks binding of IGF-I and II to the IGF receptor and has a higher affinity binding for IGF-I than does the type 1 IGF receptor (Francis *et al.* 1988; Gopinath *et al.* 1989).

The acid-labile subunit is N-glycosylated and appears as a 84/86 kDa doublet band on sodium dodecyl sulfate-polyacrylamide gels. Deglycosylation reduces the protein to its predicted size of 63 kDa (Baxter *et al.*, 1989). The importance of glycosylation status in the formation of the ternary complex has not yet been established.

The full cDNA sequence of the acid-labile subunit has been determined from a human liver cDNA library (Leong *et al.*, 1992). The mature protein encodes 578 amino acids with a 5' secretion signal of 27 hydrophobic amino acids. The sequence is largely composed of 18-20 leucine rich repeats that are typically found in a variety of proteins that participate in protein-protein interactions. Expression of the acid labile subunit is restricted to the liver and plasma

ALS levels have been shown to be regulated by GH but not IGF-I in rats (Zapf *et al.*, 1989) pigs (Walton and Etherton, 1989) and in diseased states that show altered GH secretion.

1.11 IGF-I Analogues

The biological potency of the IGFs is related to their affinity for the IGF binding proteins (Francis *et al.*, 1992). Synthetic mutants for IGF-I have been generated as analogues to determine which sequences are important for interactions with the binding proteins, and experiments have been performed to establish their altered biological potency *in vitro* and physiological effects *in vivo*. Naturally occurring analogues of IGF-I have also been studied.

A natural analogue of IGF-I, des(1-3)IGF-I has been isolated from bovine colostrum (Francis *et al.*, 1986) and foetal (Sara *et al.*, 1986) and adult (Carlsson-Skwirut *et al.*, 1986) human brain. This analogue is a truncated form of native IGF-I and lacks the three amino terminal residues of IGF-I. Des(1-3)IGF-I is more potent in stimulating DNA and protein synthesis and inhibiting protein breakdown than IGF-I and has a reduced affinity for IGFBP-3 and the type 1 IGF receptor (Francis *et al.*, 1992).

Clemmons *et al.* (1990) have demonstrated that contact between residues 3 and 4 at the amino terminus of the B chain and residues 49 to 51 of the A chain is crucial for IGF-1 binding to IGFBP-I. Mutations at these positions results in the greatest reduction in binding activity. Optimum IGF-I binding to IGFB-2 also requires the correct amino acids at these positions (Clemmons *et al.*, 1992). Nuclear magnetic resonance studies have shown that the solution structure of IGF-I has these two regions in close proximity with possible hydrogen bond interactions between them (Cooke *et al.*, 1991). These results also suggest that IGFBP-1 and -2 share similar structural conformation. In contrast, only residues in the B domain appear to be crucial for IGFBP-3 binding to IGF-I. [Gln^{3}, Ala^{4}, Tyr^{15}, Leu^{16}] IGF-I is 100 fold less potent than IGF-I, while substitutions at positions 49 to 51 in the A chain result in only a minimal loss of binding affinity (Clemmons *et al.*, 1992). These results indicate that the A chain is not required for IGFBP-3 binding and that the IGFBP-3 binding region is smaller than that of IGFBP-1 and -2. These substitutions in the B chain do not alter the affinity of the IGF-I analogue for the type 1 IGF receptor. Substitutions in the B chain of residue 24 with a nonaromatic amino acid (leucine) or replacement of residues 28 to 37 with a 4-glycine bridge produce IGF-I analogues with a 30 and 50-fold reduction in affinity for the type 1 IGF receptor without altering their affinity for IGFBP-3 (Cascieri *et al.*, 1988; Bayne *et al.*, 1989). IGF-I analogues in which residues of the A chain are substituted with the analogous residues

in the A chain of insulin have almost the same affinity for the type 1 IGF receptor and binding proteins as does native IGF-I, but altered affinity for the type 2 IGF receptor (Cascieri *et al.*, 1989). While most mutants show a decrease in receptor affinity, a [Tyr^{55}, Gln^{56}] substitution results in an IGF-I analogue with a 7-fold increased affinity for the type 2 receptor.

The critical amino acid in IGF-I for IGFBP binding is the negatively charged glutamate (Glu) at position 3. The substitution of Glu^3 with Gly^3, as discussed, places an amino acid with neutral charge at that position. However, the binding affinity of IGFBPs for IGF-I can be even further reduced by substituting the negatively charged amino acid with a positively charged arginine residue at position 3 (King *et al.*, 1992). Francis *et al.* (1992) have incorporated this substitution into an analogue that has a 13 amino acid N terminal extension, including the first 11 amino acids of porcine GH ([Met^1]-pGH(1-11)-Val-Asn-[Arg^3]-IGF-I). This analogue is termed Long R^3IGF-I (LR^3IGF-I). The N-terminal extension peptide allows the correct folding of the recombinant analogue. In addition to having a lower affinity for the binding proteins, LR^3IGF-I also has a reduced affinity for the type 1 IGF receptor but is significantly more potent than IGF-I in stimulating protein and DNA synthesis and inhibiting protein breakdown (Francis *et al.*, 1992). In normal adult rats, LR^3IGF-I is cleared much more rapidly from the circulation than IGF-I, while in pregnant rats, where the levels of IGFBP-3 are low, the clearance rate of IGF-I is similar to LR^3IGF-I which does not differ to the clearance rate observed in non-pregnant rats (Bastian *et al.*, 1993). These results indicate that while in the circulation, LR^3IGF-I remains free and does not readily bind to IGFBP-3 or form a ternary complex with the acid labile subunit. In lambs, des(1-3)IGF-I is also cleared from the circulation at a faster rate than IGF-I (Francis *et al.*, 1988) but has a greater affinity for the IGFBPs and the type 1 IGF receptor than does LR^3IGF-I.

IGF-I analogues have also been shown to be more potent than IGF-I *in vivo*. The [Gln^3, Ala^4, Tyr^{15},Leu^{16}] IGF-I analogue is more potent than IGF-I in glucose incorporation into glycogen in the rat diaphragm (Cascieri *et al.*, 1988), and both des(1-3)IGF-I and LR^3IGF-I are more potent than IGF-I in stimulating growth and metabolism of normal (Tomas *et al.*, 1993), dexamethasone-treated (Tomas *et al.*, 1992b), diabetic (Tomas *et al.*, 1991b), nitrogen-restricted (Tomas *et al.*, 1991a) or surgically compromised rats (Martin *et al.*, 1991; Lemmey *et al.*, 1991).

1.12 The Growth Hormone-Insulin-like Growth Factor Axis

Initially it was thought that the synthesis of IGF-I by the liver and its secretion is exclusive regulated by pituitary derived GH and following its release into the circulation, IGF-I then mediates the somatogenic actions in the target tissues. Subsequently it has been shown that IGF-I is produced independently of GH status in other non-hepatic tissues (D'Ercole *et al.*, 1984) This has led to an alternative concept of IGF-I action, where GH stimulates hepatic IGF-I production, as well as there being an auto/paracrine action of IGF-I (Underwood *et al.*, 1986). This has been confirmed *in vitro* in a number of endocrine glands, where other local trophic hormones regulate IGF-I levels (Williams, 1988; Hsu and Hammond, 1987).

1.12.1 Growth hormone and its effect on the GH/IGF-I axis

In mammals, the GH gene is a single gene, although in primates this gene has undergone duplication to generate a cluster of GH-like genes at a single locus. The gene is highly conserved between species and has 5 exons that are spread over approximately 2.6 to 3 kb in most mammalian species (Tuggle & Trenkle, 1996). It has been isolated from many species including rat (Page *et al.*, 1981), human (DeNoto *et al.*, 1981), cow (Woychik *et al.*, 1982) and pig (Seeburg *et al.*, 1983). Alternative splicing of the GH gene results in the generation of two mRNA species that are translated into two proteins of 22 and 20 kDa of which the former is predominant in the circulation (Baumann, 1991).

GH is produced in the anterior pituitary, released under the stimulus of growth hormone releasing factor (GRF) (Barinaga *et al.*, 1985) and inhibited by somatostatin. A variety of other factors also modulate GH secretion from the pituitary. In rat pituitary cells, triiodothyronine and cortisol stimulate GH gene expression and secretion (Martial *et al.*, 1977) while insulin and the IGFs have been shown to be inhibitory (Weber *et al.*, 1992; Yamashita and Melmed, 1986a; Abe *et al*, 1983).

There is considerable evidence that GH can regulate its own secretion via a negative feedback mechanism. In rats, systemic and intraventricular administration of GH inhibits growth hormone releasing factor (GRF) and stimulates the release of somatostatin resulting in suppression of spontaneous GH pulses (Carlsson *et al.*, 1990, Chihara *et al.*, 1981). Passive immunisation of GH treated rats with a somatostatin antiserum is able to reverse the GH induced inhibition on spontaneous GH pulses (Lanzi and Tannenbaum, 1992). GH has also

been reported to stimulate somatostatin release *in vitro* (Robbins *et al.*, 1985) as well as increasing somatostatin mRNA levels in the hypothalamus (Rogers *et al.*, 1988).

1.12.2 Effects of GRF on the GH/IGF-I axis

The effect of hypothalamic GRF on the IGF-I/GH axis was demonstrated by Armstrong *et al.* (1993). They showed that active immunisation of heifers against GRF not only decreased plasma GH levels but resulted in suppression of plasma IGF-I, IGFBP-3 and insulin, while upregulating plasma IGFBP-2.

GRF has been shown to stimulate somatostatin release from median eminence fragments (Aguila and McCann, 1988) and hypothalamus (Katakami *et al.*, 1986) *in vitro* as well as increasing somatostatin gene transcription (Zeytin *et al.*, 1988). Similarly, intracerebroventricular (icv) administration of GRF in sheep stimulates somatostatin release (Spencer *et al.*, 1992). The proposed mechanism by which this occurs is that GRF releases nitric oxide which diffuses to the adjacent somatostatin neurons where it activates guanylate cyclase, leading to increased formation of cGMP and somatostatin mRNA (Aguila, 1994).

Immunoneutralisation of GRF abolishes episodic release of GH and suppresses plasma IGF-I levels in pigs (Armstrong *et al.*, 1990), indicating that circulating IGF-I concentrations are dependent on pituitary derived factors. In the rat, GRF immunoneutralisation produces similar effects on plasma IGF-I levels, decreases pituitary GH mRNA and somatostatin mRNA levels and increases GRF. Adding GH or IGF-I to this treatment regime suppresses the increase in GRF mRNA seen with immunoneutralisation, suggesting that both hormones are important regulators of GRF synthesis (Uchiyama *et al.*, 1994).

1.12.3 Effects of IGF-I on the GH/IGF-I axis

The effect of IGF-I on somatostatin release is suggested to be through GRF (Aguila *et al.*, 1993) which regulates somatostatin gene expression as previously discussed. IGF-I receptor mRNAs are widely expressed by the endocrine cells of the anterior pituitary lobe (Bach and Bondy, 1993), hypothalamus (Goodyer *et al.*, 1984) and median eminence (Bohannon *et al.*, 1986). Intracerebroventricular administration of IGF-I to GH deficient rats decreases hypothalamic GRF mRNA expression and unlike GH, increases somatostatin mRNA levels (Sato and Frohman, 1993). IGF-I has also been implicated in the negative feedback inhibition of GH secretion (Silverman *et al.*, 1989) and mRNA at the level of the

pituitary (Yamashita and Melmed, 1986b) suggesting that both a short loop (via pituitary) and long loop (via hypothalamus) negative feedback mechanism exists for IGF-I.

1.13 Effects of GH Administration *in vivo*

1.13.1 Metabolic effects of GH treatment

Numerous studies have confirmed that administration of GH improves the rate, composition and efficiency of growth in domestic and laboratory animals. The pig has been the most extensively used model because of its agricultural significance. GH treatment increases average daily weight gain, feed : gain ratio and thus decreases the average daily feed intake (Weeden *et al.*, 1993). GH enhances protein accretion and reduces fat deposition in pigs (Campbell *et al.*, 1989). The mechanism by which it reduces fat deposition is mostly due to a reduction in lipogenesis (Dunshea *et al.*, 1992a). Increased protein deposition is achieved by increased nitrogen retention, protein synthesis and amino acid utilisation in pigs (Tomas *et al.*, 1992a). Although GH treatment in pigs also increases the rate of protein breakdown, the rate of protein synthesis is greater, leading to a net positive effect on protein accretion. GH treatment results in minimal changes of muscle fibre size in longissimus muscle while in semitendinosus muscle GH treatment increases type IIB and decreases type I fibres suggesting a shift towards a faster contractile type muscle fibre in treated animals (Lefaucheur *et al.*, 1992).

1.13.2 Effects of GH treatment on glucose metabolism

Injection of GH has been shown to cause transient hyperglycaemia and hyperinsulinaemia in pigs (Dunshea *et al.*, 1992b; Johnson *et al.*, 1990; Klindt *et al.*, 1992). The hyperglycaemic response does not occur in fasted pigs after a single intra-arterial injection (acute) of GH, but chronic administration of GH for 24 days does result in post-injection hyperglycaemia (Gopinath and Etherton, 1989a). This hyperglycaemia is more likely to be caused by a decrease in glucose uptake than an increase in glucose utilisation. During chronic GH administration, glucose uptake by adipocytes for lipogenesis (Walton *et al.*, 1987) and glucose clearance rates (Gopinath and Etherton 1989b; Wray-Cahen *et al.*, 1993) are reduced. Glucose uptake by skeletal muscle, liver and adipocytes is insulin dependent, however, steers

receiving chronic administration of bovine GH show a reduction in glucose uptake in the hind limb despite elevated insulin levels (Boisclair, 1989). These results suggest that chronic GH treatment induces a state of insulin resistance or decreases tissue sensitivity to insulin. Most of the circulating glucose is taken up by the liver and stored as glycogen (glycogenesis), which is then converted to glucose (gluconeogenesis) and released when required. Insulin stimulates glycogenesis and inhibits gluconeogenesis. Therefore a reduction in hepatic insulin sensitivity could stimulate gluconeogenesis and glycogen mobilisation resulting in hyperglycaemia. This increase in blood glucose concentrations leads to an increased release of insulin from the pancreas and combined with decreased hepatic insulin extraction, results in hyperinsulinaemia. This situation is also found in acromegalic patients who exhibit high levels of GH and insulin and show a reduced ability for insulin to inhibit gluconeogenesis (Hansen *et al.*, 1986).

1.13.3 Effects of GH treatment on organ growth

GH treatment has a selective effect on some organs. It has been shown to increase longitudinal bone growth (Isaksson *et al.*, 1982) and bone wall thickness (Evock *et al.*, 1988) indirectly by increasing IGF-I, which stimulates osteoblast proliferation. Anterior and posterior pituitary weights are increased in a dose dependent manner, while liver, adrenal, kidney, uterus, ovary and heart weights are unaffected when measured 42 days after the last GH injection (Andres *et al.*, 1993). Others have reported increased kidney, heart, ovary and adrenal weights (Evock *et al.*, 1988; Bryan *et al.*, 1989; Bryan *et al.*, 1992) when animals are slaughtered 24 hours after the last injection.

High doses of GH treatment has been associated with the formation of esophagogastric ulcers (McNamara *et al.*, 1991; Smith *et al.*, 1991; Lefaucheur *et al.*, 1992) and long term treatment with porcine GH (pGH) decreases bone strength (Goodband *et al.*, 1993). This decrease in bone strength is associated with increased leucine requirements with GH treatment and thus increased requirements for calcium and phosphorous. Adding phosphorous to the diets of GH treated animals increases bone strength without changing the GH effects on growth performance (Weeden *et al.*, 1993).

1.13.4 Effects of GH treatment on IGF-I and IGFBP-3

Many of the growth promoting effects of GH are believed to be mediated by IGF-I. It is therefore not surprising to find that GH treatment increases plasma IGF-I levels in many different species. Hypophysectomized rats treated with GH show increased levels of plasma IGF-I and IGFBP-3 (Glasscock *et al.*, 1991). Transgenic mice carrying the ovine GH gene under the regulation of the metallothioneine promoter show elevated levels of plasma IGF-I and IGFBP-3 as well as increased expression of hepatic IGF-I and IGFBP-3 upon activation of the transgene (Chow *et al.*, 1994). In contrast, Palmer *et al.* (1993) have reported that 22 day old rats injected twice daily for 10 days with GH show no changes in plasma or hepatic IGF-I mRNA levels, although whole body growth and muscle mass is increased. There is evidence that the GH/IGF-I axis does not develop until 14 - 28 days. It is therefore possible that these animals are insensitive to GH. GH also regulates the expression of IGF-I mRNA in rat skeletal muscle, heart (Isgaard *et al.*, 1989) and liver of adult (Glasscock *et al.*, 1991) and juvenile rats (Domenè *et al.*, 1993). Liver IGFBP-3 mRNA expression is also regulated by GH but appears to be less sensitive than IGF-I to circulating GH levels (Domenè *et al.*, 1993). In sheep, GH increases plasma IGF-I levels and liver IGF-I mRNA (Hua *et al.*, 1993). GH deficient patients receiving GH show that IGF-I levels are elevated 4 - 6 hours after the commencement of treatment, and patients receiving either a continuous infusion or 8 bolus injections rather than 2, exhibit higher IGF-I levels. IGFBP-3 shows a gradual increase after a lag phase of 18 - 20 hours and IGF-II levels follow a similar pattern (Jorgensen *et al.*, 1991). In the pig, GH has shown to have a similar effect. Plasma IGF-I and IGFBP-3 levels are elevated while IGFBP-2 but IGF-II concentrations are suppressed in GH treated animals (Coleman and Etherton, 1991; Owens *et al.*, 1990). With long term GH treatment (11 weeks), plasma IGF-II concentrations are elevated in pigs (Evock *et al.*, 1990) In addition, GH treatment in pigs increases the proportion of IGF-I bound to the 150 kDa complex indicating that GH also increases the expression of the acid labile subunit. At the level of gene expression, GH has been shown to regulate IGF-I in liver, skeletal muscle (Grant *et al.*, 1991) and adipose tissue (Wolverton *et al.*, 1992).

1.14 Effects of IGF Administration *in vivo*

With the advent of recombinant DNA technology it has become possible to produce milligram amounts of IGFs and administer them to animals in relatively large doses to study their effects on growth and metabolism. Rodents have been the most extensively used to study the effects of IGF administration since they are a suitable laboratory animal and their lower body weights require smaller amounts of peptide. Many studies have examined the effects of IGF treatment in restoring normal metabolic responses in sick or genetically altered animals, however few studies exist that document the effects of IGF administration to normal laboratory and domestic animals or humans.

1.14.1 Effects of IGF-I treatment on organ growth and body weight

Systemic administration of IGF-I to dwarf rats that exhibit low levels of endogenous IGF-I results in normalisation of plasma IGF-I levels and restoration of body growth (Skottner *et al.*, 1989). Subcutaneous infusion of IGF-I stimulates the growth of hypophysectomized rats (Guler *et al.*, 1988), post-weaning normal rats (Hizuka *et al.*, 1986), and Snell dwarf mice (van Buul-Offers *et al.*, 1988). Long term treatment with IGF-I reduces subcutaneous fat in obese patients (Laron *et al.*, 1992). In contrast, IGF-I has no effect on growth in pigs and LR^3IGF-I reduces average daily gain in pigs (Walton *et al.*, 1994). This negative effect of IGFs on growth has also been reported in rats, where intracerebroventricular administration of a preparation enriched for insulin-like activity resulted in an 8% decrease in body weight gain (Tannenbaum, 1983). It should be noted though that in this case, the method of administration was directly into the cerebrum rather than the circulation.

In the rat, during foetal and early postnatal life, IGF-I treatment stimulates growth, but GH does not (Philipps *et al.*, 1988). Dexamethasone induces a state of catabolism in rats. Tomas *et al.*, (1992b) have shown that IGF treatment can partially reverse the effects of dexamethasone treatment, and the analogues des(1-3)IGF-I and LR^3IGF-I are greater than two fold more effective. IGF treatment increases the levels of plasma IGFBPs, especially IGFBP-3 and decreases the rate of muscle breakdown in catabolic and diabetic rats (Tomas *et al.*, 1991a; 1991b). IGF-I infusion into normal growing rats has shown similar responses. IGF-I and LR^3IGF-I increase body weight gain, improve nitrogen retention and food conversion efficiency with LR^3IGF-I showing a more potent response (Tomas *et al.*, 1993). IGF-I treatment also affects selective organ growth. IGF-I stimulates kidney, gut, spleen and thymus

growth in hypophysectomized rats (Guler *et al.*, 1988) and stimulates erythropoiesis (Philipps *et al.*, 1988) as well as stimulating ^{3}H-thymidine incorporation into cartilage (Hizuka *et al.*, 1986).

1.14.2 Effects of IGF-I treatment on glucose metabolism

In vivo, acute doses of IGF-I cause hypoglycaemia in man (Guler *et al.*, 1987) rats (Zapf *et al.*, 1986) and pigs (Walton *et al.*, 1989b). The hypoglycaemic effect of IGF-I can be attributed to high levels of free IGF-I, which accounts for about 80% of circulating IGF-I and has a half life of approximately 20 minutes during the hour immediately following bolus administration of IGF-I. In addition, plasma IGF-I levels remain elevated for more than 7 hours following injection, while plasma glucose levels returned to normal within two hours (Guler *et al.*, 1987). This pool of IGF-I must therefore represent IGF-I bound to IGFBP-3. Hypoglycaemia may be avoided in long term infusion of IGF-I. Guler *et al.* (1989) found that infusing man with IGF-I at a rate of 20 µg/kg/hour for six days results in no changes in plasma glucose levels, and IGF-I treatment suppressed plasma levels of triglycerides and cholesterol. Concentrations of C-peptide, creatinine, urea and growth hormone are also reduced. In contrast, Turkalj *et al.* (1992) have shown that continuous IGF-I infusion at doses of 5-30 µg/kg/hour for 8 hours produces a dose-dependent increase in glucose metabolism. Plasma triglyceride, free fatty acid, C-peptide and insulin concentrations show a dose dependent decrease. Similar results have also been observed with insulin infusion (Kazumi *et al.*, 1986). That the method and length of administration determines to what extent IGF-I affects glucose homeostasis was also shown by Dimitriadis *et al.* (1992). They showed that the soleus muscle isolated from rats treated with either an acute (single dose) or chronic dose (10 day infusion) of IGF-I is able to convert glucose to hexose monophosphate and form lactate and glycogen. Animals receiving chronic doses of IGF-I are less effective, and insulin resistance is evident in animals receiving acute doses of IGF-I. These results suggest that IGF-I increases muscle glucose uptake.

It has been shown that insulin inhibits lipolysis, and that this occurs at a lower concentration than glucose uptake (Nurjhan *et al.*, 1986). The decrease in free fatty acids associated with IGF-I treatment can be associated with a decreased rate of lipolysis brought about by the insulin-like actions of IGF-I. The degree of plasma glucose suppression by IGF-I is similar to that of insulin, but the levels of free fatty acids are decreased to a greater extent by insulin (Bang and Hall, 1992).

1.14.3 Effects of IGF-I treatment on plasma hormones

Treatment with IGF-I results in altered levels of expression for GH, the acid labile subunit, the IGF binding proteins and insulin and differs between species. IGF-I has been shown to be a potent inhibitor of glucose induced insulin secretion in man (Morgan *et al.*, 1993; Rennert *et al.*, 1993) and rats (Leahy and Vandekerkhove, 1990). Glucose disposal is also accelerated by IGF-I, and the inhibitory effect on insulin can be diminished with an increased hyperglycaemic stimulus for glucose uptake (Rennert *et al.*, 1993). In humans, subcutaneous injection of 100 μg/kg once daily for 7 days resulted in increased plasma IGFBP-1 and decreased plasma IGFBP-3 and the acid labile subunit (Baxter *et al.*, 1993). Similar treatment (40 μg/kg twice daily for 7 days) of adult patients with a growth hormone receptor deficiency, and therefore low levels of IGF-I, show that IGF-I treatment normalises plasma IGF-I levels, decreases plasma IGF-II but does not change the low levels of plasma IGFBP-3 characteristic of these patients. The already elevated IGFBP-2 levels are marginally increased (Fielder *et al.*, 1993). The amount of IGF-I found associated in a 150 kDa complex increases with treatment, but is probably a result of decreased plasma IGF-II levels rather than increased expression of the acid labile subunit. In contrast, another study of patients exhibiting an absence of GH receptor activity has shown that IGF-I treatment (120-150 μg/kg for 6 months) increases plasma IGFBP-1, IGFBP-2 and IGFBP-3 levels and decreases plasma insulin levels. This effect is less evident in adults than in children (Kanety *et al.*, 1993). The difference in observed IGFBP-3 levels may be attributed to the dosage and length of administration. The elevated levels of plasma IGFBP-2 in these patients can be explained by the absence of GH action since GH is known to have a suppressive effect on IGFBP-2 expression. IGF-I treatment has been shown to increase IGFBP-2 expression, which is also evident in these GH receptor deficient patients. Insulin suppresses IGFBP-1 expression, it therefore follows that IGFBP-1 levels are elevated when plasma insulin levels are reduced. This increase in plasma IGFBP-1 has also been observed in normal patients (Baxter *et al.*, 1993).

The decrease in plasma IGFBP-3 in normal humans treated with IGF-I is difficult to explain. It may be that GH is regulating the expression of IGFBP-3, and that IGF-I treatment reduces plasma GH levels which then leads to suppressed plasma levels of IGFBP-3 and the acid labile subunit. These results are in contrast to earlier findings by Zapf *et al*, (1990). They found that IGF-I infusion, rather than injection, over a period of six days produced a marginal

increase in plasma IGFBP-3. Similar findings have been observed in the rat. Subcutaneous administration of LR^{3}IGF-I or IGF-I to catabolic or diabetic rats results in an increase in IGFBP-1 and IGFBP-3 (Zapf *et al.*, 1989 and Tomass*et al.*, 1992). In contrast, yearling lambs treated with IGF-I (50 μg/kg, 3 times daily for 8 weeks) show only a transient increase in plasma IGFBP-1 even though plasma insulin levels continue to remain suppressed. There was no change in plasma IGFBP-3 levels. Plasma IGFBP-2 levels were suppressed while plasma glucose was elevated (Cottam *et al.*, 1992).

Intraventricular administration of IGF-I to rats, suppresses plasma GH concentrations (Abe *et al*, 1983), suggesting that IGF-I acts to feedback inhibit GH secretion. In a similar study, intracerebroventricular administration of a preparation enriched for insulin-like activity results in a similar suppression of plasma GH levels (Tannenbaum, 1983). This suggests that IGF-I administered systemically is able to act directly at the tissue level to improve growth performance, whereas when administered to the brain, acts to inhibit GH secretion which may then reduce circulating IGFs and growth performance.

1.15 Combination Treatment of IGFs with GH

Very few studies have examined the effects of combined treatment of IGF-I with GH. GH as an anabolic agent is largely limited by its tendency to cause hyperglycaemia, while IGF-I treatment has a tendency to cause hypoglycemia. Combining these two agents may result in a normalisation of plasma glucose and could result in a synergistic effect in the growth promoting qualities of these proteins. In hypophysectomized neonatal rats, IGF-I is a poor promoter of somatic growth despite normalisation of plasma IGF-I levels and increased levels of IGFBP-3. GH alone is able to stimulate body weight gain without altering plasma IGFBP-3 or IGF-I levels. However combining IGF-I and GH in the same treatment does not improve body weight gain above that seen with GH treatment alone (Glasscock *et al.*, 1992). The lack of any response with either of the treatments involving IGF-I may be due to the fact that the neonatal rats used in this study were at the stage of development where the GH/IGF-I axis is not fully developed.

A study in diet restricted man has shown that IGF-I/GH combination treatment results in greater nitrogen retention than IGF-I treatment alone, an attenuation of IGF-I related hypoglycemia by GH and conversely an attenuation of hyperglycaemia and hyperinsulinaemia observed with GH treatment alone. Plasma IGF-I concentrations are higher in the combination treatment than for IGF-I treatment alone and plasma IGFBP-3 and acid labile subunit levels

are also elevated for the combined treatment. Interestingly, IGF-I treatment alone causes a significant decrease in both plasma IGFBP-3 and acid labile subunit concentrations (Kupfer *et al.*, 1993). In this example of catabolism, a combination treatment with IGF-I and GH has been shown to be successful at reversing nitrogen wasting and thus increasing protein accretion in muscle and connective tissue. The addition of GH to IGF-I treatment results in increased expression of IGFBP-3 and acid labile subunit which in turn increase the half life of IGF-I and decrease the insulin suppressive effects. The higher levels of insulin can enhance the anabolic effects of IGF-I by inhibiting proteolysis (Fukagawa *et al.*, 1985). In turn IGF-I is able to prevent the insulin resistance seen with GH treatment. These studies show promising results for administering IGF-I in combination with GH to produce synergistic effects on growth performance in both normal and catabolic states.

CHAPTER 2

AIMS OF THE CURRENT STUDY

IGF-I and LR^3IGF-I treatment has been shown to reverse catabolic states in dexamethasone treated rats (Tomas *et al.*, 1992b) and promote growth in normal rats (Tomas *et al.*, 1993). In contrast, observations by Walton *et al.* (1994) have shown that pigs treated with IGF-I do not show enhanced growth performance and those treated with LR^3IGF-I exhibit a reduction in average daily gain. There are a number of hypotheses that could be put forward to explain these findings. One of these is that the IGF peptides may be acting to reduce GH secretion or synthesis and that this reduction in plasma GH may affect plasma levels of components of the IGF-I/GH axis.

Evidence in the rat suggests that inhibition of GH by systemically administered IGF-I occurs through negative feedback at the pituitary (Sato and Frohman, 1993), although a direct effect at the hypothalamus can not be rule out since intracerebroventricular (icv) infusion of IGF-I stimulates somatostatin mRNA and inhibits GRF mRNA expression (Sato and Frohman, 1993).

GH treatment has been shown to increase IGF-I mRNA expression in various porcine tissues. To the end of 1993, investigations into the tissue specific expression of IGF-I, and GH regulation of IGF-I *class 1* and *class 2* mRNA expression were limited. At that time we knew that in the pig, GH treatment increased IGF-I expression in liver, skeletal muscle (Grant *et al.*, 1991) and adipose tissue (Wolverton *et al.*, 1992) and IGF-I *class 2* transcripts had only been detected in the liver (Weller *et al.*, 1993). To what extent GH regulates IGF-I *class 1* and *class 2* mRNA in different organs was not known. In addition, no study had investigated the tissue specific regulation of GH-dependent porcine IGFBP-3 mRNA expression. The hypothesis to be tested was that the poor growth response observed in pigs was due to negative feedback effects of IGFs on GH secretion and/or expression which decreases the expression of endocrine regulated IGF-I and/or IGFBP-3. This in turn would lead to a reduction in plasma IGF-I and IGFBP-3 levels and consequently a reduction in growth. To this extent, the current study will investigate plasma IGF-I, IGFBP-3 and GH levels in pigs treated with IGF-I or LR^3IGF-I; determine the expression of porcine IGFBP-3 and IGF-I in a range of tissues collected from GH-treated pigs; and quantify the expression of IGF-I and IGFBP-3 in GH-responsive tissues of IGF-I and LR^3IGF-I treated pigs using porcine probes specific for IGFBP-3 and IGF-I *class 1* and *class 2* mRNA.

Combination treatment of IGFs with GH has shown positive results in humans by reducing the hypoglycaemic effects of IGF-I administration and increasing nitrogen retention (Kupfer *et al.*, 1993). This has led to the question whether combination treatment in pigs can ameliorate the negative growth performance seen in LR^3IGF-I treated pigs. Thus growth performance, plasma levels of components of the IGF-I/GH axis and IGF-I and IGFBP-3

mRNA levels will be measured to determine if combination treatment results in any interactive effects between IGF and GH treatment.

The aims of this study are as follows:

1. To determine if the lack of growth performance in IGF-I or LR^{3}IGF-I treated pigs is associated with a feedback inhibition on GH secretion or synthesis.
2. To determine if the lack of growth performance in IGF-I or LR^{3}IGF-I treated pigs is associated with reduced levels of plasma IGF-I and IGFBP-3
3. To determine in which tissues IGF-I *class 1, class 2* and IGFBP-3 mRNA expression is regulated by GH.
4. To determine if IGF-I or LR^{3}IGF-I treatment results in decreased expression of endocrine regulated IGF-I and/or IGFBP-3.
5. To determine if combination treatment of IGF-I or LR^{3}IGF-I with GH has an interactive effect on growth performance in pigs.

CHAPTER 3

CONTINUOUS 4 DAY INFUSION OF LONG [R^3] INSULIN-LIKE GROWTH FACTOR-I (LR^3IGF-I) REDUCES GROWTH, PLASMA GROWTH HORMONE, IGFBP-3 AND ENDOGENOUS IGF-I CONCENTRATIONS IN PIGS.

3.1 Introduction

Growth hormone (GH) has been demonstrated to improve growth performance in the pig (Etherton *et al*, 1986; Campbell *et al*, 1988, Klindt *et al.*, 1992). The negative side effects of pGH treatment include hyperglycaemia and hyperinsulinaemia due to development of insulin resistance (Dunshea *et al.*, 1992b). It has been postulated that insulin-like growth factor-I (IGF-I) mediates the effects of GH in lean tissue growth but not in fat deposition (Thompson *et al.*, 1995). Consequently, IGF-I has been suggested as an alternative growth promoting agent since it does not produce the hyperglycaemia observed during GH treatment (Rennert *et al.*, 1993). Both IGF-I and GH treatment increase whole body protein synthesis in rats (Lo *et al.*, 1995), man (Mauras, 1995) and pigs (Malmlöf *et al.*, 1995).

LR^{3}IGF-I is a analogue of IGF-I with a N-terminal extension comprising of the first 11 amino acids of methionyl-pGH linked via a Val-Asn bridge to the full human IGF-I sequence that has a Arg for Gly substitution at position 3. It is more potent than IGF-I in cell culture because it has a low affinity for IGF binding proteins (Francis *et al.*, 1992) and improves growth performance *in vivo* in rats (Tomas *et al.* 1992b and 1993). In contrast, IGF-I has little effect, and LR^{3}IGF-I reduces growth in pigs (Walton *et al.*, 1994).

Combined IGF-I and GH treatment has been shown to have an additive effect on body weight recovery in neonatal catabolic rats (Zhao and Donovan, 1995) and enhance the lipolytic and ketogenic effects of GH in normal human subjects (Bianda *et al.*, 1996) There is however no added benefits on whole body protein anabolism with combination treatments in normal human subjects (Mauras, 1995).

In the present study, I examined if the decline in growth rate in the pig is associated with changes to the GH/IGF-I axis by measuring plasma concentrations of IGFBP-3, IGF-I, insulin and GH after infusion of IGF-I or LR^{3}IGF-I. To determine if the negative effects of IGF peptides on growth performance could be reversed by concurrent treatment with pGH, similar analyses were performed on plasma samples obtained from pigs receiving IGF-I or LR^{3}IGF-I in combination with pGH.

3.2 Materials and Methods

3.2.1 Peptides and radionucleotides

Recombinant human IGF-I and LR^3IGF-I were supplied by GroPep Pty. Ltd., Adelaide, SA, Australia. Porcine GH was supplied by Bresatec Ltd, Adelaide, SA, Australia. Porcine insulin was supplied by Eli Lilly Company, Indianapolis, IN, USA. Growth hormone releasing factor (GRF) was supplied by Auspep Pty. Ltd., Parkville, Victoria, Australia. Porcine IGFBP-3 and a rabbit antiserum for porcine IGFBP-3 had been developed in this laboratory by Dr. Paul Walton. $Na\text{-}I^{125}$ was obtained from Amersham Australia Pty Ltd., NSW, Australia.

3.2.2 Animals and animal maintenance

Pigs were obtained from and were housed at the Victorian Institute of Animal Science, Werribee, Victoria, Australia. The animals were individually penned in metabolism cages throughout the experiment and were fed 110g/kg body weight per day split into 3 meals given at 0800, 1200 and 1600 hours.

3.2.3 Experimental design

Twenty four crossbred boars (55 kg) were surgically prepared with indwelling catheters in the anterior vena cava under halothane (May & Baker, West Footscray, Victoria, Australia) anaesthesia 8 days prior to commencement of the experiment. This time span allowed for recovery from surgery and acclimatisation of the environment. At surgery, all pigs received an intra muscular injection of a long acting antibiotic, oxytetracycline (May & Baker, West Footscray, Victoria, Australia) and received medicated feed (1g/kg terramycin, May & Baker, West Footscray, Victoria, Australia) throughout the study. Pigs were grouped according to liveweight, and within groups, were randomly allocated in a 3 x 2 factorial experimental design with the respective factors being growth factor (vehicle, IGF-I or LR^3IGF-I) and growth hormone (saline or pGH). Growth factors were infused at a rate of 180 μg/kg/day for 90 hours via Alzet miniosmotic pumps (Alza Corporation, Palo Alto, CA, USA) inserted subcutaneously into the neck. Growth hormone was administered at a dose of 30 μg/kg/day in four subcutaneous injection. Vehicle treatments were acetic acid (100 mmol/l)

for IGF peptides and NaCl (150 mmol/l) for pGH. Feed intake and body weights were measured daily and blood samples were taken at the following times: -48, -40, -24, -18, 0 (immediately prior to pump insertion), 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 56, 64, 72, 80, 88, 96, 108, 120, 132, and 144 hours. For acute measures of GH secretion, blood samples were taken every 10 minutes for 6 h before treatment and from 88 h to 94 h of the treatment period. After 94 hours of treatment, all animals were challenged with an intravenous dose of 1.8 μg/kg GRF, and blood samples were taken 3, 6, 10, 20, 30, 35 and 60 minutes after the injection. Plasma was separated by centrifugation and stored at -20°C until analysed.

The study was approved by the Victorian Institute of Animal Science Animal Experimentation Ethics Committee and the Animal Ethics Committee from the University of Adelaide.

3.2.4 Filling and insertion of pumps

Alzet model 1003D miniosmotic pumps were filled with either 100 mM acetic acid (vehicle), 39 mg of IGF-I or 39 mg of LR^3IGF-I. This provided an infusion rate for the IGF peptides of 10 mg per day. Pigs were snared with a snout rope and anaesthetised locally with an injection of Lignocaine (May & Baker, West Footscray, Victoria, Australia) behind the left ear. A troca, 5 mm in internal diameter, was inserted subcutaneously behind the ear and the osmotic pump inserted under the skin. A single stitch was used to seal the wound.

3.2.5 Determination of average daily gain and feed intake

Pigs were weighed daily at 8 am on commercial livestock scales that had been incorporated into a metabolism cage. To determine feed intake, the amount of feed remaining after every 24 hour period was weighed and subtracted from the total daily feed supplied.

3.2.6 Iodination of proteins

To prepare proteins for iodination, 10 μg lyophilised human IGF-I, porcine GH or porcine insulin was dissolved in 50 μl 10 mM HCl (porcine GH was dissolved in 50 μl 25 mM Tris, pH 8.0) for 30 minutes at room temperature and diluted in an equal volume of 0.5 M sodium phosphate (pH 7.5). Iodination was performed by the chloramine-T method (Proudman & Wentworth, 1978) using 1 mCi NaI^{125} (Amersham Australia Pty Ltd., NSW, Australia) and 20 μl 0.4 mg/ml chloramine-T (Sigma Chemical Company, St. Louis, MO, USA) for 60 seconds. The iodination reaction was terminated by the addition of 20 μl 0.6 mg/ml sodium metabisulphite (Sigma Chemical Company, St. Louis, MO, USA) and incubated at room temperature for 2 minutes. ^{125}I labelled proteins were separated by chromatography through a Sephadex G50 (Pharmacia Biotech, Uppsala, Sweden) column (1 cm x 25 cm) in a buffer containing 50 mM sodium phosphate, 150 mM sodium chloride and 2.5 g/l BSA (pH 6.5). The flow rate was set at 0.25 ml/minute and 500 μl fractions were collected. All fractions were counted on a gamma counter to determine the amount of radioactivity and fractions containing the radioactive protein peaks were precipitated with 10% trichloroacetic acid (TCA). Those fractions containing ^{125}I-IGF-I with greater than 95 % TCA precipitable protein were retained, aliquoted and stored at -20°C.

3.2.7 Determination of plasma IGF-I concentration

3.2.7.1 Separation of IGF peptides from IGFBPs in plasma by acid gel-filtration chromatography

To prepare the samples for chromatography, 40 μl of plasma was diluted with 260 μl of sterile water and mixed with 100 μl of a solution of 0.8 M acetic acid and 0.2 M trimethylamine (pH 2.5). An equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (freon) was mixed thoroughly with the acidified plasma solution in order to remove lipids. The solution was centrifuged at 10, 000 x *g* for 10 minutes at 4°C to separate the freon and plasma phases. A 300 μl aliquot of the plasma solution was then subjected to gel-filtration chromatography under acid conditions using a Waters Protein Pak 125 column (7.8 mm x 30 cm; Waters, Lane Cove, NSW, Australia) to collect the IGF peptides as a distinct and separate pool from the IGF binding proteins. The separation buffer (mobile phase) consisted of 0.2 M acetic acid, 50 mM trimethylamine and 0.05 % (v/v) Tween 20 (pH 2.5). The flow rate was 1 ml per minute.

To ensure that the positions of the elution pools were consistent throughout the period of chromatography, the elution volume of chromatographed ^{125}I-labelled IGF-I was compared at regular intervals. To determine which eluate fractions to pool for measurements of plasma IGF-I concentrations, the fractions collected following chromatography of plasma pools from vehicle, IGF-I and IGF-II treated rats were assayed by IGF-I radioimmunoassay.

3.2.7.2 IGF-I radioimmunoassay (RIA)

Plasma IGF-I concentrations were calculated by RIA analysis of the neutralised IGF pools generated by the chromatography step described in section 3.2.7.1.

In a final volume of 460 µl, the assay contained 100 µl chromatography eluate or mobile phase (standard or reference tubes), 60 µl 0.4 M Tris, 50 µl rabbit, anti-IGF-I polyclonal antiserum (GroPep Pty Ltd., Adelaide, SA, Australia), 50 µl ^{125}I-IGF-I (25,000 cpm) and 200 µl RIA buffer or 200 µl human IGF-I reference standard (19.5 pg - 10 ng/ml). The RIA buffer consisted of 0.2 M sodium phosphate, 0.5 M EDTA, 0.02 % (w/v) protamine sulfate and 0.05 % (v/v) Tween 20, pH 7.5. Assays were set up on ice and left to incubate overnight at 4°C. Assays were completed by the addition of 50 µl sheep, anti-rabbit γ globulin (Silenus Laboratories Pty Ltd., Melbourne, Victoria, Australia) and 10 µl rabbit-γ-globulin (Dako, CA, USA). Incubation proceeded for 30 minutes at 4°C followed by the addition of 1 ml cold 5.5% (w/v) polyethylene glycol 5000 and 0.9 % (w/v) sodium chloride. The tubes were centrifuged at 4,000 x *g* for 20 minutes at 4°C, the supernatant was aspirated and the radioactivity in the pellet determined using a gamma counter. Each sample was assayed in triplicate.

3.2.8 IGFBP-3 radioimmunoassay

A covalent complex of ^{125}I-IGF-I and IGFBP-3 was used as a tracer in the IGFBP-3 RIA. Human IGF-I was iodinated as described in section 3.2.6. The IGFBP-3/IGF-I complex was generated by the addition of 1 µg iodo-IGF-I (0.22 mCi) to 8 µg pure IGFBP-3 dissolved in 1 ml column buffer (50 mM sodium phosphate; 150 mM sodium chloride; 2.5 g/l BSA, pH 6.5) and incubated at 22°C for 2 h. A solution of 0.25 M disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL, USA) in 10 µl dimethylsulfoxide was added and incubated for a further 30 min at 22°C. The reaction was terminated by the addition of 50 µl 1 M Tris-HCl,

pH 8.0 and free ^{125}I-IGF-I was separated from the IGFBP-3/IGF-I complex by chromatography on a 1 x 40 cm Sephadex G-100 (Pharmacia Biotech, Uppsala, Sweden) column, equilibrated and eluted with 0.5 M acetic acid; 0.1 M NaCl; 0.25% (w/v) BSA, pH 3.0.

For preparation of plasma, a 20 µl aliquot of porcine plasma was acidified with an equal volume of 0.8 M acetic acid, 1.2% (v/v) trimethylamine, pH 2.8, 2% (v/v) Tween-20 and incubated at room temperature for 20 min. Samples were then neutralised with 0.6 volumes 0.4 M Tris, diluted to 1 ml, and 50 µl used for the IGFBP-3 RIA.

In a reaction volume of 500 µl, the assay contained 50 µl of pig plasma or 50 µl standard (0.2 - 40 ng/ml), 100 µl IGFBP-3 antiserum, and 100 µl of IGFBP-3/^{125}I-IGF-I complex (10,000 cpm). The volume was adjusted with assay buffer containing 50 mM sodium phosphate, 150 mM NaCl, 1 % BSA (w/v), 0.02 % sodium azide (v/v), pH 7.2. Tubes were incubated overnight at room temperature. The assay was completed by the addition of 100 µl 1 % normal rabbit serum and 100 µl of goat anti-rabbit IgG (Silenus Laboratories Pty Ltd., Melbourne, Victoria, Australia). Incubation proceeded at room temperature for 30 minutes followed by the addition of 1 ml ice-cold 6 % polyethylene glycol. The tubes were centrifuged at 4000 *x g* for 20 minutes at 4°C, the supernatant aspirated and the radioactivity in the pellet counted using a gamma counter. Each sample was assayed in triplicate.

3.2.9 Insulin radioimmunoassay

Porcine insulin was iodinated as described in section 3.2.6. Plasma insulin concentrations were determined using a guinea pig, anti-porcine insulin antisera (Sigma Chemical Company, St Louis, MO, USA). In a reaction volume of 400 µl, the assay contained 100 µl of pig plasma or 200 µl porcine insulin standard (19.5 pg - 10 ng/ml), 100 µl of charcoal stripped plasma and 50 µl insulin antiserum. The volume was adjusted with assay buffer containing 50 mM sodium phosphate, 0.5 % BSA (w/v), 0.01% sodium azide (v/v), pH 7.4. Tubes were incubated for 3 days at 4°C. A 50 µl aliquot of ^{125}I-insulin (20 000 cpm) was added and the assay was incubated for a further 24 hours at 4°C. The assay was completed by the addition of 100 µl of donkey anti-guinea pig antibody coated cellulose suspension (Wellcome Reagents Ltd., Beckenham, UK). Incubation proceeded at room temperature for 30 minutes followed by the addition of 500 µl of glass distilled water. The tubes were centrifuged at 4000 x *g* for 10 minutes at 4°C, the supernatant aspirated and the radioactivity in the pellet counted using a gamma counter. Each sample was assayed in triplicate.

3.2.10 Growth hormone radioimmunoassay

Iodination of porcine GH was performed as described in section 3.2.6. In a final volume of 500 µl, the assay contained 50 µl of pig plasma or 200 µl pGH standard (0.46 ng - 40 ng/ml) and 50 µl rabbit, anti-porcine growth hormone antiserum (UCB Bioproducts, Belgium) The volume was adjusted with assay buffer containing 0.01 M phosphate buffered saline, 0.1 % BSA (w/v), 0.01% sodium azide (v/v), pH 7.4. A 50 µl aliquot of ^{125}I-pGH (20 000 cpm) was added and the assay was incubated at room temperature overnight. The assay was completed by the addition of 50 µl donkey anti-rabbit antibody coated cellulose suspension (Wellcome Reagents Ltd., Beckenham, UK). Incubation proceeded at room temperature for 30 minutes followed by the addition of 500 µl of glass distilled water. The tubes were centrifuged at 4000 x *g* for 10 minutes at 4°C, the supernatant aspirated and the radioactivity in the pellet counted using a gamma counter. All samples were assayed in triplicate.

3.2.11 Determination of plasma glucose concentration

To determine plasma glucose concentrations, 30 µl of plasma was aliquoted into sampling vials and assayed using a Cobas Fara II autoanalyser (Hoffman LA Roche Ltd., Switzerland). To ensure consistency throughout the assay, a known rat plasma sample was included in the assay at the beginning and end of each carousel. All samples were assayed in duplicate.

3.2.12 Statistical Analysis

All data was analysed using Genestat 5 Release 3.1. Average daily gain (ADG), feed intake, feed : gain ratio, plasma IGF-I, IGFBP-3 and plasma glucose were analysed in a 2 x 3 factorial design two way analysis of variance, with the respective factors being pGH (0 or 30 µg/kg/day) or IGF (0, IGF-I or LR^{3}IGF-I at 180 µg/kg/day). Significant differences were determined using Bonferroni's method of comparison. Feed : gain analysis was performed on transformed data because of some negative growth rates. Data was transformed using the equation (1000 x feed intake)/(ADG + 1000). Plasma insulin was averaged over the treatment period and data was analysed using repeated measures one way analysis of variance. Significant differences were determined using Bonferroni's method of analysis. Insulin data

was also analysed using the same 2 x 3 factorial experimental design two way analysis of variance. Pretreatment values were used as a covariate.

Growth hormone profiles were analysed using PULSAR (Merriam & Wachter, 1982), with a smoothing window of 360 min and minimum pulse interval of 30 min. Baxter parameters were determined using the equation $y = B1 + B2x + B3x^2$ in triplicate on 7 different dose levels of pGH. The threshold value for peak acceptance was set at (in standard deviation units): 4.4, 2.60, 1.92, 1.46 and 1.13 for pulses of 10, 20 30 40 and 50 minutes duration, respectively. For GH profiles, peak number, peak amplitude, baseline GH concentration, average GH concentration and area under the GH spikes were analysed using a 2 x 3 factorial experimental design two way analysis of variance with the respective factors being pGH (0 or 30 µg/kg/day) or IGF (0, IGF-I or LR^3IGF-I at 180 µg/kg/day). Pretreatment values were used as a covariate. Where there was a significant difference between pretreatment values, the treatment values were adjusted for the covariate. Significant differences were determined using Bonferroni's method of comparison.

3.3 Results

3.3.1 Growth performance

Average daily weight gain (ADG), feed intake and feed : gain ratio during 4 days of treatment are shown in Table 3.1. Statistical analysis of the data show that ADG and feed : gain were increased by pGH. LR^3IGF-I significantly decreased ADG compared to IGF-I but was not different to animals receiving neither growth factor. Feed intake was reduced by LR^3IGF-I but the feed : gain ratio was unaffected by growth factor treatment. Combination treatment with pGH did not alter the effects of growth factor treatment on ADG or feed intake. There were no differences in ADG, feed intake and feed : gain ratio in the post treatment period.

Table 3.1:

Effect of vehicle, IGF-I or LR^3IGF-I infusion alone or in combination with pGH injection on ADG, feed intake and feed : gain ratio

	GH			IGF				Significance[1]		
	saline	pGH	*sed*[2]	control	IGF-I	LR^3IGF-I	*sed*[3]	G	I	G x I
ADG (g/day)	663	933*	*107*	812	975	606[b]	*131*	0.023	0.041	0.636
Feed intake ($g/kg^{0.75}$/day)	104.5	101.5	*4.7*	108.9	108.7	91.4*	*5.7*	0.523	0.011	0.546
Feed : gain ratio	64.5	52.6**	*3.3*	60.4	55.8	59.5	*4.0*	0.003	0.499	0.083

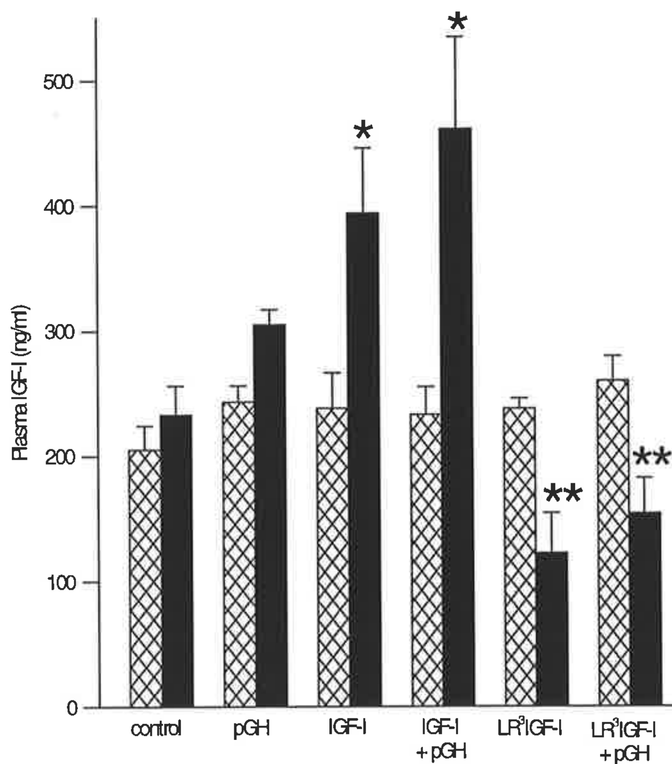
*Significant differences from the vehicle group are denoted by * P < 0.05; ** P < 0.005. A significant difference from the IGF-I group is denoted by [b] P < 0.05. [1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I); [2]- standard error of the difference of means for GH (G), n = 12; [3]- standard error of the difference of means for growth factor (I), n = 8; [4] - since there were some negative growth rates, the analysis was conducted on transformed data, where transformed feed : gain = (1000 x feed intake)/(ADG + 1000)*

3.3.2 Hormone concentrations

Plasma IGF-I concentrations were measured at the start and after 72 h of treatment. (Figure 3.1). As expected, groups receiving IGF-I showed an increase in plasma IGF-I levels. In contrast, endogenous plasma IGF-I levels were significantly decreased by both LR^3IGF-I treatment alone, and LR^3IGF-I in combination with pGH.

Figure 3.1:

Plasma IGF-I concentration measured at the start (hatched bars) and after 72 hours (solid bars) of treatment

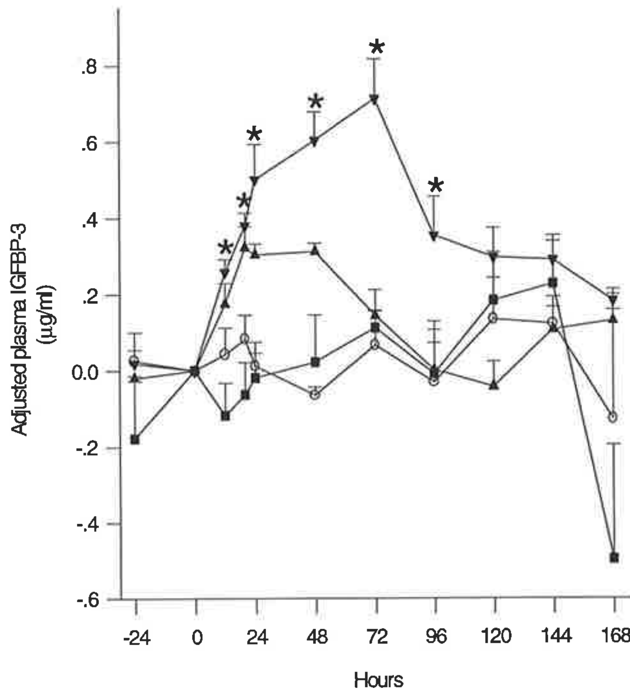


*Results are expressed as the mean ± SEM for 4 animals in each treatment group. Significant differences are denoted by * indicating a significant increase in plasma IGF-I compared to the vehicle treated group (P < 0.05) and ** indicating a significant decrease in plasma IGF-I compared to the vehicle treated group (P < 0.05).*

Figures 3.2 and 3.3 illustrate the difference in plasma IGFBP-3 concentration compared to values obtained at the start of treatment. Infusion of IGF-I alone had no effect on plasma IGFBP-3. In combination with pGH, IGF-I significantly elevated plasma IGFBP-3 after 20 h of treatment (Figure 3.2). In contrast, LR^3IGF-I infusion decreased plasma IGFBP-3 concentration after 72 h followed by a return to control values by 2 days post-treatment. Growth hormone was unable to reverse the reduction in plasma IGFBP-3 caused by LR^3IGF-I infusion (Figure 3.3).

Figure 3.2:

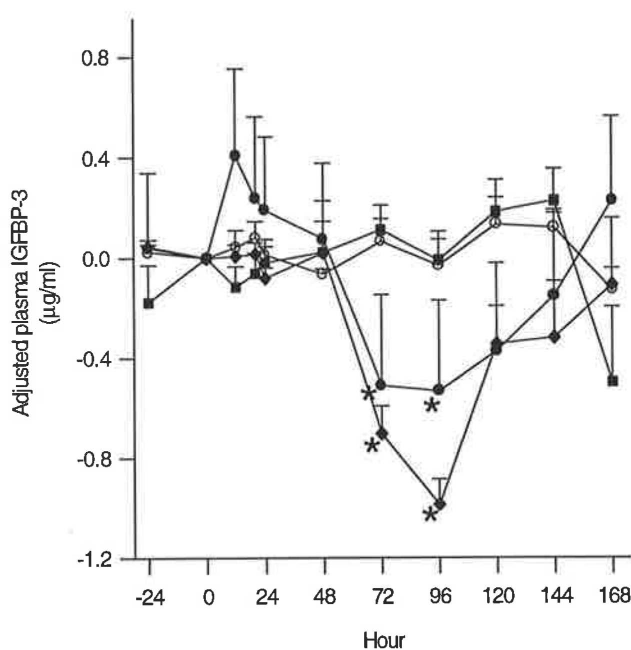
Effect of vehicle (○), pGH (■), IGF-I (▲) or IGF-I + pGH (▼) treatment on changes in porcine plasma IGFBP-3 concentrations compared to the averaged pre-treatment value



*Results are expressed as the mean ± SEM for 4 animals in each treatment group. Averaged pre-treatment value across all treatment groups for plasma IGFBP-3 concentration was (1.61 ± 0.45 µg/ml). Significant differences from pretreatment values are denoted by * $P < 0.05$.*

Figure 3.3:

Effect of vehicle (○), pGH (■), LR^3IGF-I (◆) and LR^3IGF-I + pGH (●) on changes in plasma IGFBP-3 concentrations compared to the average pre-treatment value

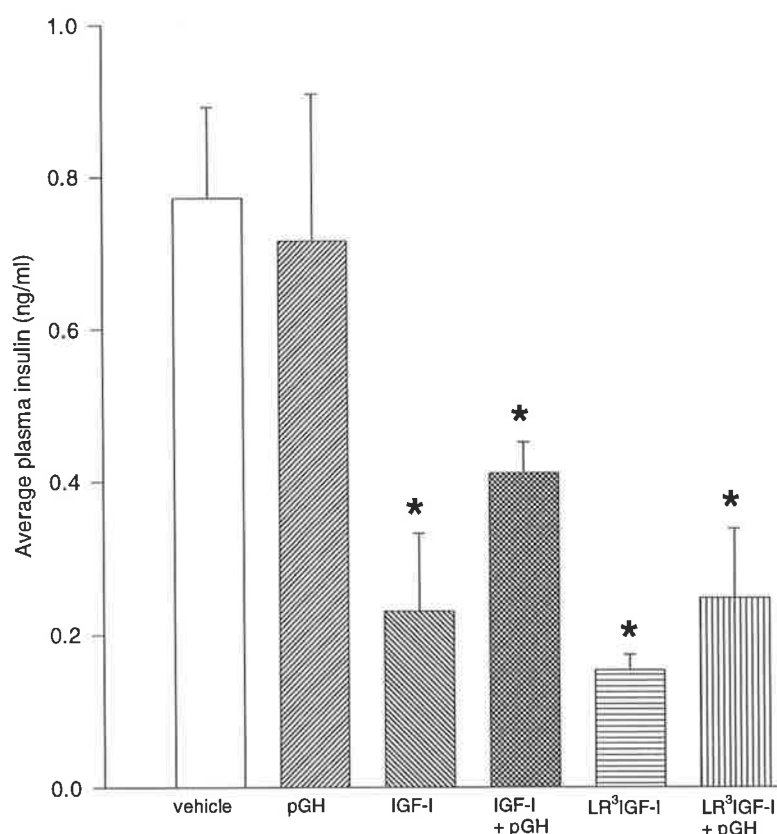


*Results are expressed as the mean ± SEM for 4 animals in each treatment group. Averaged pre-treatment value across all treatment groups for plasma IGFBP-3 concentration was (1.61 ± 0.45 µg/ml). Significant differences from pre-treatment values are denoted by * $P < 0.01$.*

Average plasma insulin concentrations during 72 hours of treatment are shown in Figure 3.4. Growth hormone had no effect on plasma insulin concentration whereas IGF-I and LR^{3}IGF-I significantly reduced plasma insulin concentration. Combination treatment with growth hormone was unable to reverse the reduction in plasma insulin concentrations caused by growth factor treatment. There was no interactive effect between growth hormone and growth factor treatments as analysed by a 2 x 3 factorial design two way ANOVA with growth factor or GH as variables and using pre-treatment values as a co-variate (Table 3.2).

Figure 3.4:

Porcine plasma insulin concentration averaged over 72 hours of treatment.



*Values are mean ± SEM (n = 4). Data was analysed using repeated measures one-way ANOVA. Significant differences were determined using Bonferroni's method of comparison. * denotes a significant decrease in plasma insulin concentration compared to vehicle treated animals (P < 0.05).*

Table 3.2:

Effect of vehicle, IGF-I or LR^{3}IGF-I infusion alone or in combination with pGH injection on plasma insulin concentration

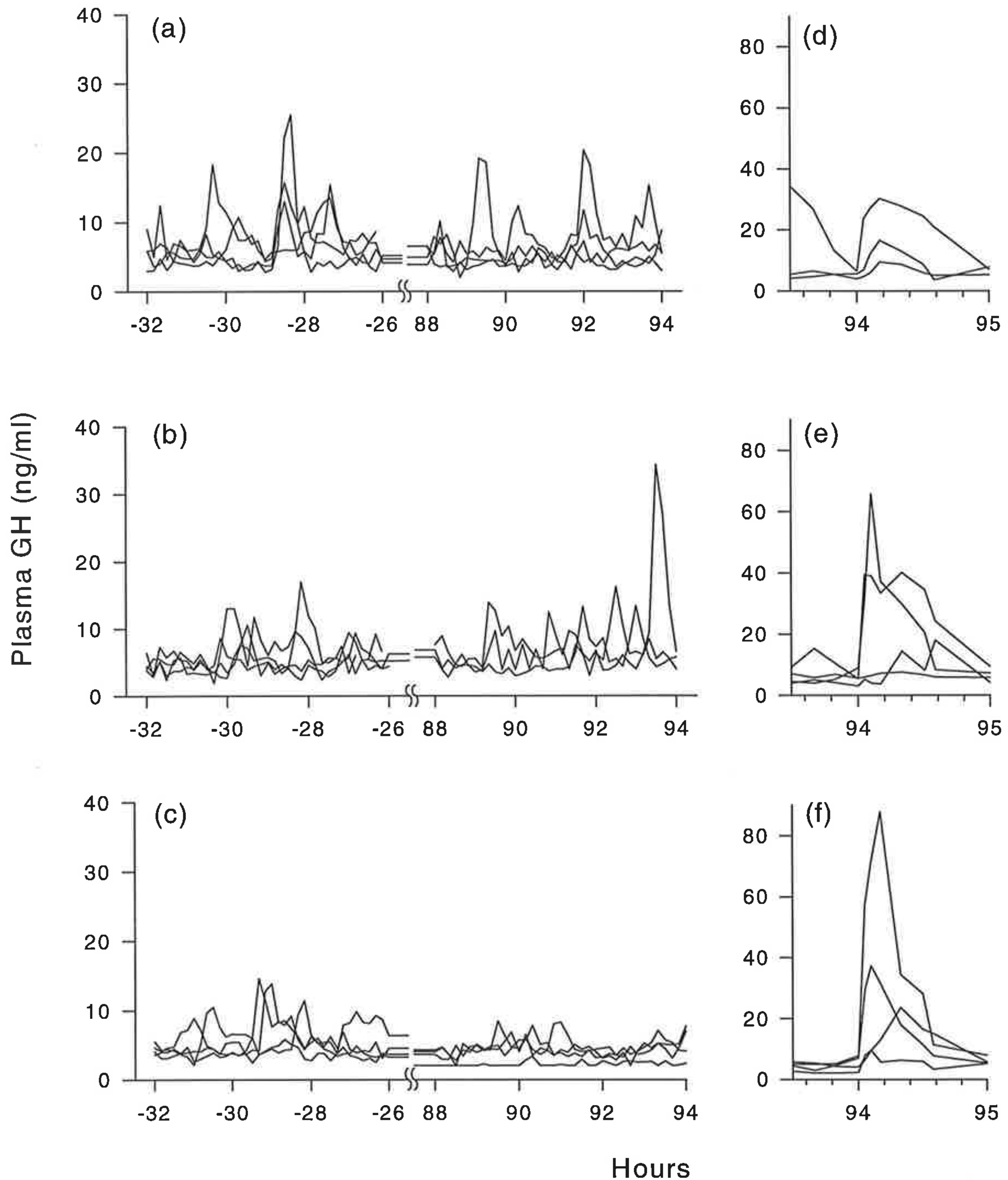
	GH			IGF				Significance[1]		
	saline	pGH	*sed*	control	IGF-I	LR^{3}IGF-I	*sed*	G	I	G x I
Plasma insulin (ng/ml)	0.245	0.292	*0.071*	0.466	0.143	0.196	*0.087*	0.474	0.003	0.832

Data was analysed using a 2 x 3 factorial design two way ANOVA with growth factor or GH as variables. Pretreatment values were used as covariate values, and the means adjusted for covariate values. This analysis was performed to establish if there was an interaction effect between growth hormone and growth factor treatment (G x I). [1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I).

Individual GH profiles from pigs treated with IGF-I or LR^{3}IGF-I are shown in the left hand panels of Figure 3.5. The plasma GH response to a GRF stimulus is presented in the right hand panels of Figure 3.5. Individual GH profiles of animals receiving the combination treatment are shown in the left hand panels of Figure 3.6 and the plasma GH response to a GRF stimulus is shown in Figure 3.6. Statistical analysis of this data is presented in Tables 3.3 and 3.4.

Figure 3.5:

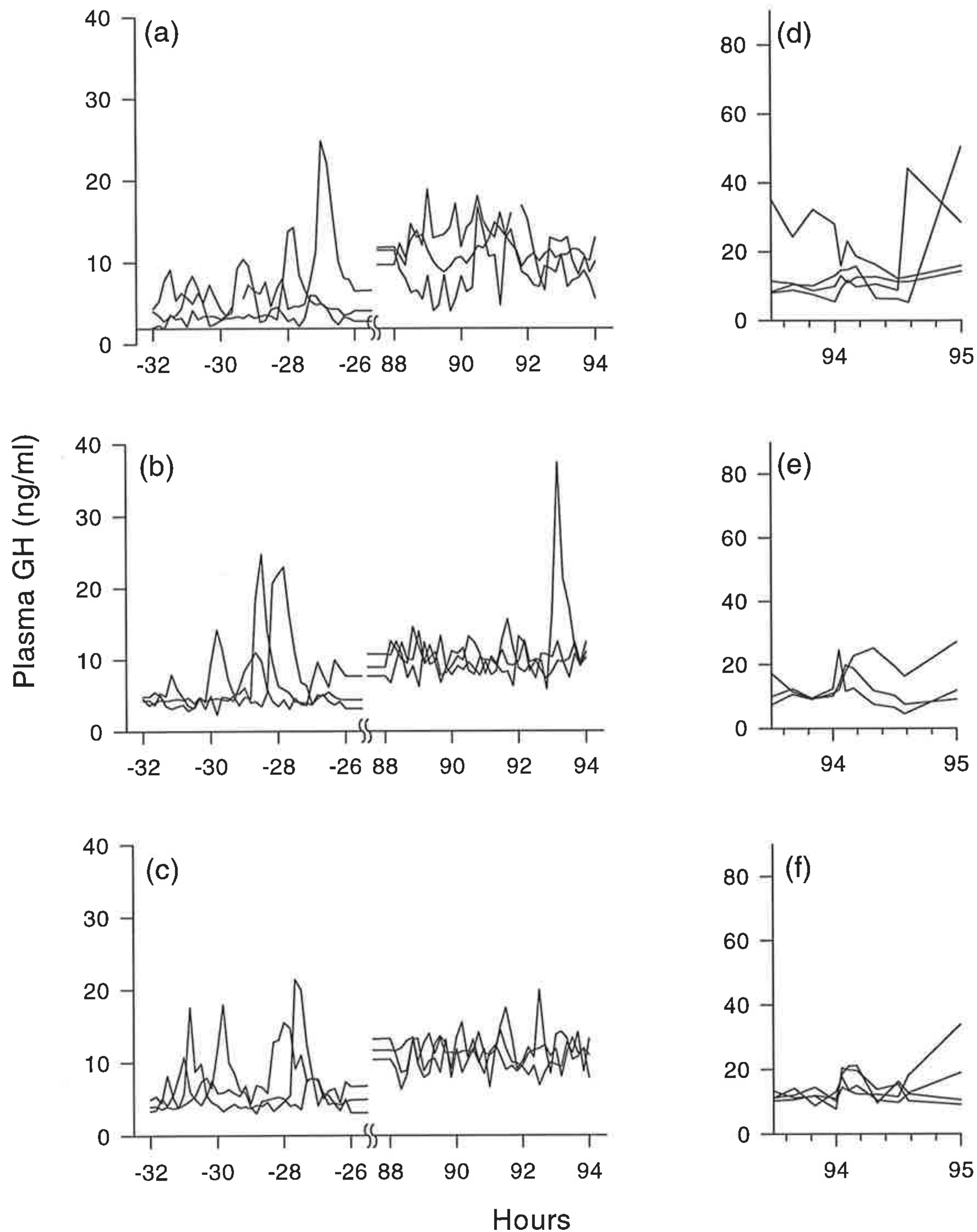
Individual plasma GH profiles from pigs treated with vehicle (a & d), IGF-I (b & e) or LR^{3}IGF-I (c & f)



Samples were obtained at 10 minute intervals for 6 hours prior to treatment and on the fourth day of treatment. After 94 hours of treatment, pigs received a single injection of 1.8 μg/kg GRF and blood samples were taken 3, 6, 10, 20, 30, 35 and 60 minutes after the injection. Plasma GH profiles during this time period for vehicle (d), IGF-I (e) and LR^{3}IGF-I (f) treated animals are shown.

Figure 3.6:

Individual plasma GH profiles from pigs treated with pGH (a & d), IGF-I + pGH (b & e) or LR³IGF-I + pGH (c & f)



Samples were obtained at 10 minute intervals for 6 hours prior to treatment and on the fourth day of treatment. After 94 hours of treatment, pigs received a single injection of 1.8 μg/kg GRF and blood samples were taken 3, 6, 10, 20, 30, 35 and 60 minutes after the injection. Plasma GH profiles during this time period for pGH (d), IGF-I + pGH (e) and LR³IGF-I + pGH (f) treated animals are shown.

The mean values for peak number, peak amplitude, baseline GH concentration, average GH concentration and area under the GH spikes adjusted for covariate values are presented in Table 3.3. Growth hormone peak number and amplitude were not affected by any of the treatments. IGF-I had no significant effect on baseline plasma GH concentration or pulse peak area but significantly decreased the mean plasma GH concentration. LR^3IGF-I treatment significantly decreased mean plasma GH concentration and pulse peak area. Growth hormone treatment increased mean and baseline GH concentrations, and combination treatment with pGH did not alter the effects of IGF-I or LR^3IGF-I treatment on mean GH concentrations and pulse peak area.

Table 3.3:

Effect of vehicle, IGF-I or LR^3IGF-I infusion alone or in combination with porcine GH injection on pulsatile GH expression

	GH			IGF				Significance[1]		
	saline	pGH	*sed*[2]	control	IGF-I	LR^3IGF-I	*sed*[3]	G	I	G x I
Peak number	7.04	6.38	*0.58*	7.21	7.03	5.89	*0.67*	0.29	0.13	0.56
Peak amplitude	3.18	5.35	*1.67*	5.90	4.00	2.91	*1.78*	0.24	0.27	0.95
Baseline plasma GH (ng/ml)	4.33	9.35**	*0.43*	7.68	6.35	6.48	*0.54*	< 0.001	0.05	0.16
Average plasma GH (ng/ml)	5.71	11.22**	*0.67*	10.05	7.67*	7.67*	*0.83*	< 0.001	0.016	0.92
Area under GH peaks (ng.h/ml)	557	501	*124*	786	492	308*	*145*	0.612	0.015	0.76

*Peak number, peak amplitude, baseline plasma GH, average plasma GH and area under the GH peaks were measured. Pretreatment values were used as covariate values, and the means have been adjusted for covariate values. Significant differences from the vehicle group are denoted by * P < 0.05; ** P < 0.001*

[1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I)

[2]- standard error of the difference of means for GH (G), n = 12

[3]- standard error of the difference of means for growth factor (I), n = 8

The effect of a single injection of GRF on the GH profiles of treated animals is shown in the right hand panels of Figure 3.5 and 3.6. The mean values for peak amplitude, baseline GH concentration and area under the GH spikes are presented in Table 3.4. Neither IGF-I or LR^3IGF-I had a significant effect on plasma GH concentrations in response to GRF, although a trend towards an increase in the area under the GH spike was evident. GH treatment significantly increased baseline GH concentrations and decreased the area under the GH spike.

Table 3.4:

Effect of vehicle, IGF-I or LR^3IGF-I infusion alone or in combination with porcine GH injection on pulsatile GH expression in response to a single injection of GRF

	GH			IGF				Significance[1]		
	saline	pGH	*sed*[2]	control	IGF-I	LR^3IGF-I	*sed*[3]	G	I	G x I
Peak amplitude	25.5	9.00	*7.95*	11.9	19.0	20.8	*9.74*	0.06	0.63	0.50
Baseline plasma GH (ng/ml)	4.64	10.26**	*0.79*	8.00	7.28	7.07	*0.94*	< 0.001	0.60	0.89
Area under GH spike (ng.h/ml)	522	211*	*139*	254	466	378	*170*	0.04	0.47	0.54

*Peak amplitude, baseline plasma GH, and area under the GH peaks were measured. Pretreatment values were used as covariate values, and the means have been adjusted for covariate values. Significant differences from the vehicle group are denoted by * $P < 0.05$; ** $P < 0.001$*

[1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I)

[2]- standard error of the difference of means for GH (G), n = 12

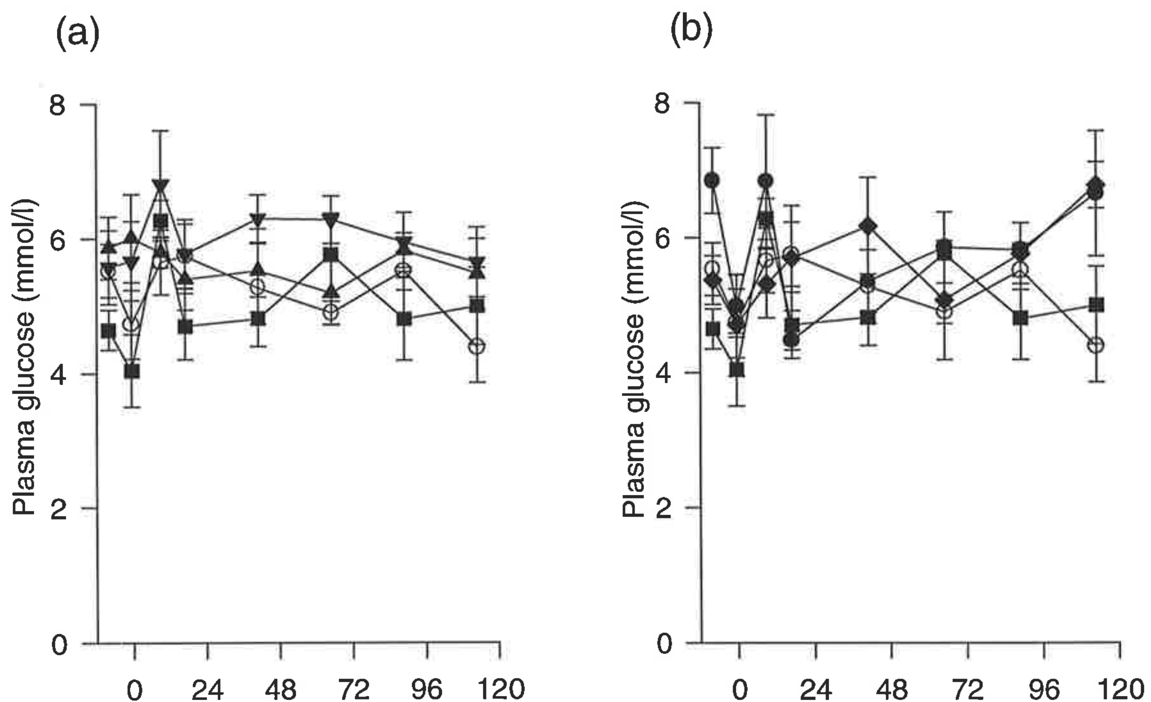
[3]- standard error of the difference of means for growth factor (I), n = 8

3.3.3 Plasma glucose

Plasma glucose concentration was measured in blood samples obtained before the first feed of each day, except on day 0 when an additional bleed was taken at 12:00, at the commencement of treatment. Plasma glucose levels were unaffected by any of the treatments (Figure 3.7).

Figure 3.7:

Plasma glucose concentrations in pigs treated with vehicle (○), pGH (■), (a) IGF-I (▲), IGF-I + pGH (▼), and (b) LR^{3}IGF-I (◆) or LR^{3}IGF-I + pGH (●)



No significant differences were observed in plasma glucose levels in animals receiving IGF-I or LR^{3}IGF-I alone (a) or in combination with pGH (b). Results are expressed as the mean ± SEM for 4 animals per treatment group.

3.3.4 Summary of results

A summary of the results obtained from the study presented in this chapter is given in Table 3.5

Table 3.5:

Effect of IGF-I and LR³IGF-I treatment alone and in combination with pGH on growth performance and plasma metabolites

	IGF-I	IGF-I + pGH	LR³IGF-I	LR³IGF-I + pGH	pGH
ADG	→	→	↓[1]	→	↑
Feed intake	→	→	↓	→	→
Feed : gain	→	→	→	→	↓
Plasma IGF-I	↑	↑	↓	↓	→
Plasma IGFBP-3	→	↑	↓	↓	→
Plasma insulin	↓	↓	↓	↓	→
Plasma glucose	→	→	→	→	→
Plasma GH	↓	↑[2]	↓	↑[2]	↑
AUC	→	→	↓	↓	→

Treatment groups are presented in the first row and parameters measured in the first column; → indicates no significant difference from vehicle treated pigs; ↓ indicates significant decrease from vehicle treated pigs; ↑ indicates significant increase from vehicle treated pigs; [1] indicates a significant decrease compared to IGF-I treated animals and [2] indicates an increase only due to exogenous pGH.

3.4 Discussion

Subcutaneous administration of IGF-I or LR^3IGF-I to normal rats promotes a dose-dependent increase in body weight (Tomas *et al.*, 1993), with LR^3IGF-I producing a more potent response. In contrast, IGF-I treatment does not stimulate growth in pigs (Walton *et al.* 1994) or sheep (Min *et al.*, 1996) and LR^3IGF-I treatment results in a decrease in growth rate in pigs (Walton *et al.* 1994). The reason for these contrasting effects is not clear, but may be related to a different hypoglycaemic or endocrine response to infusion of these peptides in the rodent, compared to other species.

Short term infusion or bolus injection of IGF-I in the human (Guler *et al.*, 1987) rat (Zapf *et al.*, 1986) and pig (Walton *et al.*, 1989b) results in hypoglycaemia. The hypoglycaemic effect of IGF-I may be attributed free IGF-I in the circulation before incorporation into the ternary complex with the acid labile sub-unit (ALS) and IGFBP-3 (Baxter *et al.*, 1989; Lewitt *et al.*, 1994). On the other hand, hypoglycaemia may not be a consequence of longer term infusion of IGF-I. Guler *et al.* (1989) found that infusion of IGF-I into humans at a rate of 20 μg/kg/hour for six days does not decrease plasma glucose concentration. Similarly, in the current study, infusion of IGF-I or LR^3IGF-I alone or in combination with pGH did not affect plasma glucose concentration in these pigs and suggests that the poor growth response is unlikely to be attributable to a hypoglycaemic effect of the peptides. The present results suggest that inhibition of components of the GH/IGF-I axis by infusion of IGF-I, and particularly, the analogue LR^3IGF-I, may be responsible for decreased growth rates in pigs.

Treatment with IGF-I results in increased lipid oxidation by releasing free fatty acids from adipose tissue. This is a consequence of reduced insulin levels which are associated with IGF-I treatment (Hussain *et al.*, 1994). In addition, IGF-I treatment leads to increased insulin sensitivity (Hussain *et al.*, 1994).These observations are supported by the present study, where IGF-I and LR^3IGF-I treatment reduced plasma insulin concentrations.

Growth hormone is the major regulator of post-natal growth in mammals. Pituitary GH secretion is regulated in a coordinated manner by episodic release of GH releasing factor (GRF) and somatostatin from the hypothalamus (Plotsky & Vale 1985; Tannenbaum, 1989). In man, somatostatin has been shown to reduce the amplitude and frequency of GH peaks, but pulsatile episodes could still be detected (Calabresi *et al.*, 1996). Evidence suggests that IGF-I also regulates plasma GH concentration. Plasma GH concentrations are suppressed during IGF-I infusion in man (Bermann *et al.*, 1994) and sheep (Fletcher *et al.*, 1995). In the present

study, both IGF-I and LR^3IGF-I decreased plasma GH concentrations in the pig, consistent with feedback inhibition at the hypothalamus and/or pituitary.

Upon administration of a GRF challenge, GH treated pigs showed a significant increase in the area under the GH spike. Neither IGF-I or LR^3IGF-I treatment had a significant effect on GH release from the pituitary following the GRF challenge. This may be due to findings that IGF-I treatment of normal humans suppresses the GH response to exogenous GRF (Bermann *et al.,* 1994).

Decreased plasma GH concentration in response to LR^3IGF-I infusion is accompanied by a decrease in endogenous plasma IGF-I concentration. This reduction in plasma IGF-I is not seen in rats treated with LR^3IGF-I (Tomas *et al.*, 1993), where plasma IGF-I levels remain within the normal range. The decrease in GH and IGF-I in response to LR^3IGF-I in the pig may be directly responsible for the decline in growth rate and may also explain the difference in growth response between the rat and the pig.

In order to determine if GH could reverse the effects of the growth factors, experiments were performed in which pigs were infused with IGF-I or LR^3IGF-I at the same time as receiving porcine GH. Growth hormone injected at a concentration of 30 µg/kg/day resulted in an increase in the basal and mean GH concentration but was unable to reverse the change in plasma GH due to IGF-I or LR^3IGF-I infusion. Previous studies in calorie restricted, healthy male volunteers have shown that GH attenuates the hypoglycaemic effects of IGF-I and the GH/IGF-I combination treatment results in increased concentrations of plasma IGF-I and IGFBP-3 (Kupfer *et al.,* 1993). In hypophysectomized rats, the combination treatment increases body weight gain when administered over 28 days, and elevates plasma IGFBP-3 levels (Fielder *et al.*, 1996). In this study, plasma IGF-I and IGFBP-3 levels were increased in response to the combined GH and IGF-I treatment, whereas IGF-I alone had no effect on plasma IGFBP-3 levels. In contrast, both IGF-I and IGFBP-3 concentrations were suppressed when LR^3IGF-I was administered alone or in combination with pGH.

It is not clear if IGF-I or GH is the primary regulator of plasma IGFBP-3. Studies in the rat suggest that IGFBP-3 expression is regulated by IGF-I (Zapf *et al.* 1989). IGFBP-3 mRNA is stimulated by IGF-I in both human and bovine fibroblast cell lines (Bale & Conover, 1992). Other studies in the hypophysectomized, neonatal rat and normal adult rat show an increase in IGFBP-3 concentration with IGF-I treatment (Glasscock *et al.* 1992; Tomas *et al.* 1992b; Fielder *et al.*, 1996). In the human, acute IGF-I injection in fasted subjects has little effect on plasma IGFBP-3 whereas longer term injections of IGF-I reduces plasma IGFBP-3 levels (Baxter *et al.*, 1993). The current study in the pig demonstrated that IGFBP-3 concentration was unaffected by IGF-I infusion unless GH was administered at the same time,

suggesting that GH is the primary regulator of IGFBP-3. In contrast, LR^3IGF-I reduced plasma IGFBP-3 levels probably as a consequence of inhibition of endogenous GH and IGF-I concentrations. Co-administration of pGH at 30 µg/kg body weight could not reverse this effect. Similar effects are seen in the guinea pig, in which administration of LR^3IGF-I resulted in a reduction in plasma IGFBP-3 (Conlon *et al.*, 1995).

In conclusion, the differences in growth seen in rats or pigs treated with LR^3IGF-I is likely to be due to the GH insensitivity found in rats. GH mediates the majority of its growth promoting effects through IGF-I. In rats, doses of 2 mg/kg/day of GH are required to elicit a growth response in rats (Hazel *et al.*, 1994) whereas in pigs, doses as low as 50 µg/kg/day are able to enhance growth performance (Chung *et al.*, 1985). This suggests that in rats, the growth promoting actions of IGF-I may not be very sensitive to GH status.

In rats, IGF-I inhibits GH release from pituitary cells *in vitro* (Yamashita & Melmed, 1986b), however exogenous LR^3IGF-I has little effect on endogenous levels of IGF-I, and indeed, plasma IGFBP-3 levels are increased in the rat (Tomas *et al.*, 1992b). In the pig, LR^3IGF-I reduces plasma GH, IGF-I and IGFBP-3 concentrations. This difference may partly explain the different growth response to LR^3IGF-I in the two species. This reduction in endogenous protein levels may be responsible for the reduction in growth observed in pigs treated with LR^3IGF-I. Whether the reduction in plasma IGF-I and IGFBP-3 levels is associated with a reduction in their mRNA expression remains to be established.

CHAPTER 4

GENERATION OF RIBOPROBES AND OPTIMISATION OF RNASE PROTECTION ASSAY

4.1 Introduction

The experiments reported in the previous chapter suggest the poor growth performance of pigs treated with LR^3IGF-I is associated with a reduction in plasma GH, IGF-I and IGFBP-3 as well as insulin. It was postulated that the reduction in plasma GH was due to decreased secretion and/or synthesis of GH from the pituitary, and this may lead to a decrease in IGF-I and or IGFBP-3 gene expression resulting in lower plasma levels of these proteins.

To allow the analysis of IGF-I and IGFBP-3 gene expression in different porcine tissues, it was necessary to obtain or construct suitable probes for use in RNase protection assays and to optimise the amount of RNA to use in the assays to allow for quantitation of changes in gene expression. This chapter describes the IGF-I probes used and the generation of the IGFBP-3 probe. Experiments performed to determine the optimum amount of RNA to be used in each assay are also presented.

4.2 Materials and Methods

4.2.1 DNA plasmids

pRP1 (Figure 4.1) and pRP2 (Figure 4.2) were kindly donated by Dr Stewart Gilmour, (Babraham Institute, Cambridge, UK) (Weller *et al.*, 1993). These vectors were constructed to generate sense or antisense riboprobes for the detection of porcine IGF-I *class 1* and *class 2* mRNA respectively.

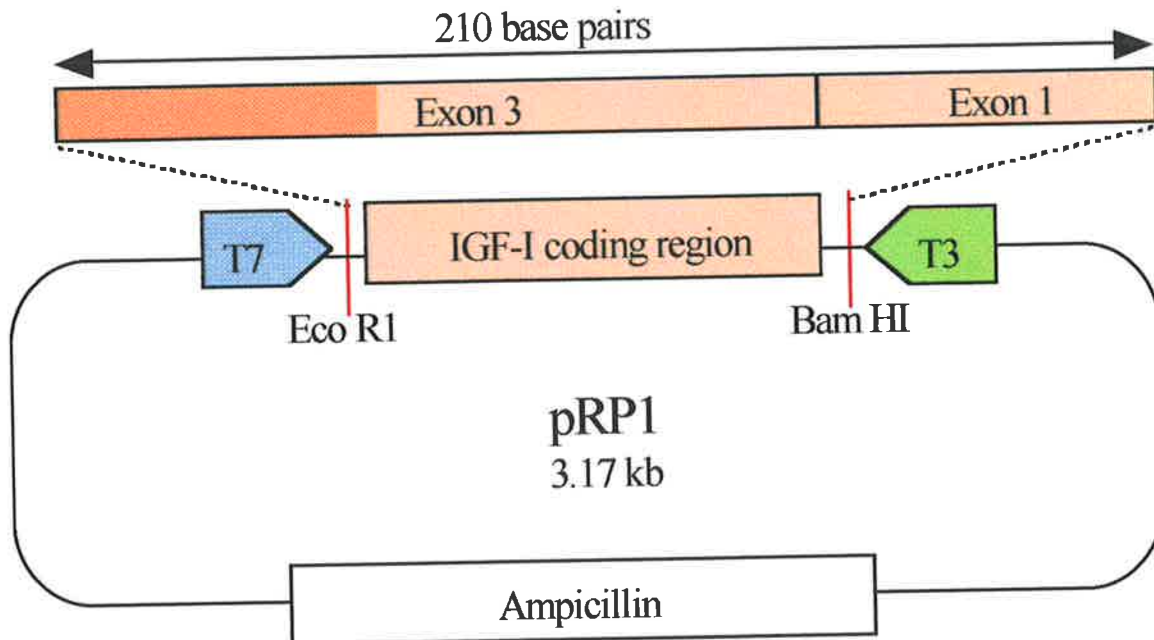
pPBP3ES (Figure 4.3) was kindly donated by Dr Shunichi Shimasaki (Whittier Institute, La Jolla, California, USA) (Shimasaki *et al.*, 1990) and contains the cDNA sequences encoding all but the first 13 amino acids of exon 1, exon 2 and the 5' end of exon 3 of porcine IGFBP-3.

pT7 RNA 18S (Figure 4.4) was purchased from Ambion Inc., Austin, Texas, USA. This plasmid serves as a antisense control template and contains a 80 base pair fragment of a highly conserved region (nucleotides 715 - 794) of the human ribosomal RNA gene.

pBluescript® II SK+ was purchased from Stratagene Ltd (Cambridge, UK). This is a transcription vector containing the T7 and T3 RNA polymerase promoters which flank a multiple cloning region. Sense or antisense riboprobes can be generated by utilising either of the promoters flanking the cloned genes.

Figure 4.1:

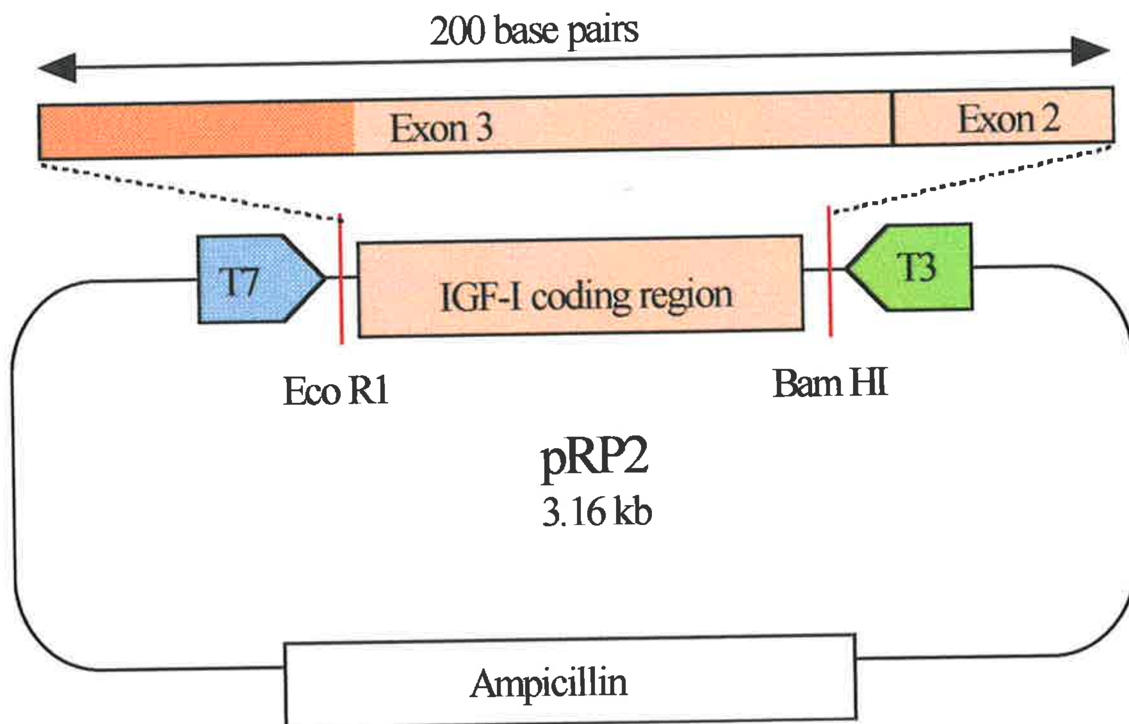
Schematic representation of plasmid vector pRP1



The ampicillin gene allows for selection of transformed bacterial colonies by conferring antibiotic resistance to bacterial cells that have been transformed with the plasmid. A 210 base pair (bp) insert extending from 53 bases upstream of the 3' end of exon 1 to 67 bases into the coding sequence of the mature IGF-I protein in exon 3 was generated by PCR and cloned into the Eco R1/Bam HI sites of Bluescript® II SK+ (Weller et al., 1993). The signal peptide sequence is indicated in pale orange, while the region encoding the mature protein is indicated in dark orange. Digestion with Bam HI and transcription from the T7 promoter produces a 270 nucleotide riboprobe.

Figure 4.2:

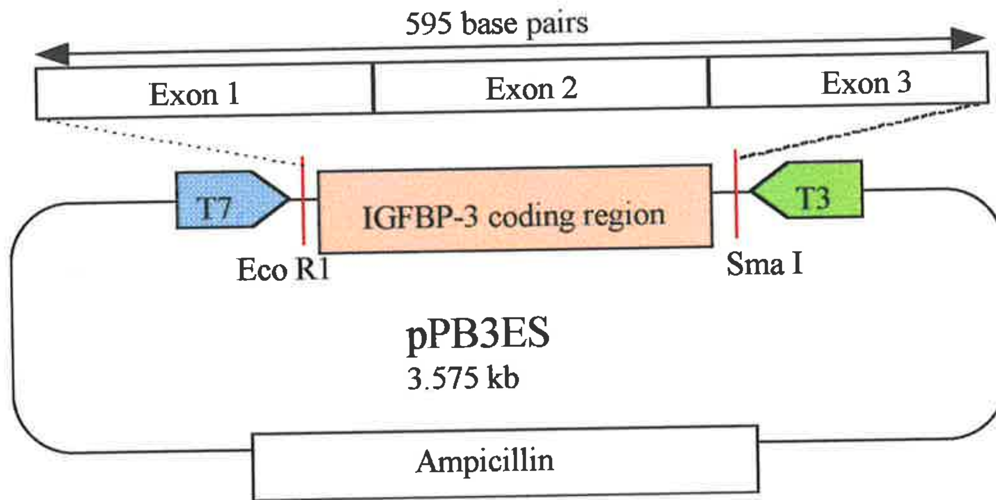
Schematic representation of plasmid vector pRP2



The ampicillin gene allows for selection of transformed bacterial colonies by conferring antibiotic resistance to bacterial cells that have been transformed with the plasmid. A 200 bp insert extending from 28 bases upstream of the 3' end of exon 2 to 67 bases into the coding sequence of the mature IGF-I protein in exon 3 was generated by PCR and cloned into the Eco R1/Bam HI sites of Bluescript® II SK+. The signal peptide sequence is indicated in pale orange, while the region encoding the mature protein is indicated in dark orange. Digestion with Bam HI and transcription from the T7 promoter produces a 260 nucleotide riboprobe.

Figure 4.3:

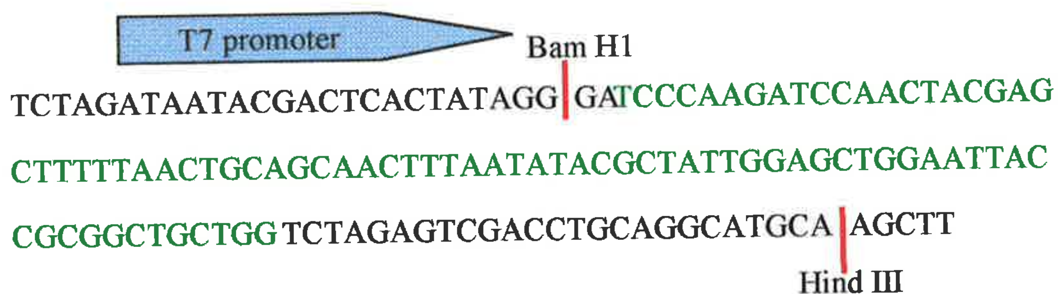
Schematic representation of plasmid vector pPBP3ES



The ampicillin gene allows for selection of transformed bacterial colonies by conferring antibiotic resistance to bacterial cells that have been transformed with the plasmid. A 595 base pair fragment extending from 39 bases upstream 5' of the first amino acid of mature porcine IGFBP-3 to 67 bases into exon 3 was cloned into the Eco R1/Sma I sites of Bluescript® II SK+.

Figure 4.4:

DNA sequence for human ribosomal RNA encoded in pT7 RNA 18S



The DNA sequence of the human ribosomal RNA gene contained within pT7 RNA 18S is indicated in green. Transcription from the T7 promoter produces a 109 nucleotide riboprobe, of which 80 bases are complementary to 18S ribosomal RNA.

4.2.2 Bacterial Strains

DH5α is a *Escherichia coli (E. coli)* bacterial strain suitable for generating cDNA libraries and subcloning. Its genotype is *deo*R, *end*A1, *gyr*A96, *hsd*R17(r_k^- m_k^+), *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ(*lac*ZYA-*arg*FV169), Φ80δ*lac*ZΔM15, F^-, λ^-. The Φ80δ*lac*ZΔM15 marker provides α-complementation of the β-galactosidase gene, allowing for blue/white colour selection of non-recombinant and recombinant colonies. *rec*A provides for insert stability of recombinants and *end*A1 improves the quality of plasmid DNA. DH5α is a restriction negative (*hsd*R17) bacterial strain and was purchased from Clontech Laboratories Inc., Palo Alto, California, USA.

4.2.3 Enzymes

The restriction enzymes Sal I, Sma I, Pst 1, Bam HI and Eco R1 were obtained from Progen Industries Ltd., Darra, Australia. Proteinase K, RQ1 RNase-free DNase and rRNasin were obtained from Promega Corporation, Madison, WI, USA. Lysozyme, RNase A and RNase T1 were obtained from Sigma Chemical Company, St. Louis, MO, USA.

4.2.4 Radionucleotides

α-^{35}S dATP, α-^{32}P UTP and γ-^{32}P dATP were obtained from Bresatec Ltd, Adelaide, Australia.

4.2.5 Chemicals

All chemicals unless otherwise indicated were of analytical reagent grade and were obtained from Sigma Chemical Company, St. Louis, MO, USA, Merk Pty Ltd, Kilsyth, Victoria, Australia or Ajax Chemicals, Auburn, NSW, Australia.

4.2.6 Small scale isolation of plasmid DNA

A single bacterial colony was inoculated into 2 ml Luria-Bertani medium (L-broth) (1% w/v bacto-tryptone, 0.05% w/v bacto-yeast extract, 0.17 M NaCl) containing 100 µg/ml ampicillin (Difco Laboratories, Detroit, MI, USA) and incubated overnight in a 37°C shaking incubator. The culture was poured into a sterile 1.5 ml Eppendorf tube and the cells pelleted

by centrifugation at 12,500 x *g* for 1 minute. The supernatant was aspirated and the pellet resuspended by vortexing in 100 μl of an ice cold solution of 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0. The cells were left at room temperature for 5 minutes and then lysed with 200 μl fresh 0.2 M NaOH and 1% SDS at 4°C for 5 minutes. Cellular debris was precipitated by the addition of 150 μl ice cold 3 M potassium acetate, vortexed and stored at 4°C for 5 minutes. The cell debris was pelleted by centrifugation at 12,500 x *g* for 5 minutes at 4°C. The supernatant was removed to a fresh tube and the plasmid DNA was purified by the addition of an equal volume phenol : chloroform (1:1), the solution vortexed, centrifuged at 12,500 x *g* at room temperature for 5 minutes and the upper aqueous phase was carefully removed to a fresh tube. This was followed with an extraction using an equal volume of chloroform. The DNA was precipitated in the presence of 0.3 M sodium acetate and 2.5 volumes 100% ethanol and centrifuged at 12,500 x *g* for 15 minutes at 4°C. The pellet was washed in 70% ethanol, dried in a vacuum centrifuge and resuspended in 40 μl TE buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA, pH 8.0).

4.2.7 Large scale preparation of plasmid DNA from bacteria

A single bacterial colony was inoculated into 50 ml L-broth containing 100 μg/ml ampicillin and incubated overnight in a 37°C shaking incubator. The culture was transferred to a 50 ml sterile tube and centrifuged at 4000 x *g* for 5 minutes at 4°C. The supernatant was aspirated and the bacterial pellet resuspended in 3 ml TES buffer (20 mM Tris, pH 7.4, 0.1 mM EDTA-Na_2, 10 mM NaCl). Bacteria were lysed with 100 mg/ml lysozyme for 5 minutes at 4°C and then with 6 ml of a freshly prepared solution of 0.2 M NaOH and 1% SDS. The tube was gently inverted several times and incubated at 4^0C for 5 minutes. Cellular debris was precipitated for 15 minutes at 4°C with 4.5 ml 3 M sodium acetate, pH 5.2 followed by centrifugation at 4000 x *g* for 10 minutes at 4^0 C. The supernatant was removed, mixed with 8 ml isopropanol and incubated at room temperature for 5 minutes. The solution was centrifuged at 4000 x *g* for 10 minutes at 4°C and the pellet drained and resuspended in 1 ml sterile glass distilled water (H_2O). To the solution was added 2 ml 5M LiCl, mixed and incubated at 4°C for 15 minutes. The solution was centrifuged at 4000 x *g* for 10 minutes at 4°C and the supernatant transferred to a fresh tube. To the supernatant was added 6 ml 100% ethanol, mixed and incubated at -20°C for 30 minutes. The plasmid DNA was pelleted by centrifugation at 4000 x *g* for 15 minutes at 4°C and the pellet drained and resuspended in 400 μl TE buffer. RNA and protein were degraded with the addition of 20 μg RNase A and

incubation at 37°C for 30 minutes, followed by the addition of 8 µl 10% SDS and 2.5 µl 20 mg/ml Proteinase K and incubation at 37°C for 15 minutes. The plasmid DNA was purified by the addition of an equal volume phenol : chloroform (1:1), the solution vortexed, centrifuged at 12,500 x *g* at room temperature for 5 minutes and the upper aqueous phase carefully removed to a fresh tube. This step was repeated and followed with an extraction using an equal volume of chloroform. The DNA was precipitated in the presence of 0.3 M sodium acetate and 2.5 volumes 100% ethanol and centrifuged at 12,500 x *g* for 15 minutes at 4°C. The pellet was washed in 70% ethanol, dried in a vacuum centrifuge and resuspended in 200 µl TE buffer.

4.2.8 DNA transformation into bacterial cells

A single bacterial colony was inoculated into 2 ml L-broth and incubated overnight in a 37°C shaking incubator. The overnight culture was diluted into 50 ml L-broth and incubated in a 37°C shaking incubator until the cells had reached log growth phase (A_{600} = 0.3 - 0.5). The cells were centrifuged at 4°C for 10 minutes at 2000 x *g* and the pellet washed in 20 ml ice cold 100 mM $CaCl_2$ and repelleted. The pellet was gently resuspended in 2 - 4 ml ice cold 100 mM $CaCl_2$ and brought up to 12 ml with ice cold 100 mM $CaCl_2$.. The cells were incubated on ice for 30 minutes and pelleted at 2000 x *g* for 10 minutes at 4°C. The pellet was gently resuspended in 2 ml ice cold 100 mM $CaCl_2$ and stored on ice. Approximately 100 ng of plasmid DNA was mixed with 200 µl of competent cells and incubated on ice for 45 minutes. The cells were heat shocked for 2 minutes at 42°C, and 1 ml L-broth added to the cells. After incubation at 37°C for 1 hour, cells were pelleted for 30 seconds at 12,500 x *g*, resuspended in 100 µl L-broth and spread onto agar plates (0.17 M NaCl; 1% bacto-tryptone, 0.5% yeast extract, 1.5% agar, 100 mg/ml ampicillin) using sterile technique.

4.2.9 Storage of bacteria

For long term storage, bacteria from inoculated overnight cultures were stored as 40% glycerol stocks at -80°C. Short term storage of bacteria was as agar plate stocks at 4°C. Bacteria were streaked out onto agar plates, incubated at 37°C overnight and stored at 4°C for approximately 4 weeks.

4.2.10 Agarose gel electrophoresis

All DNA samples were run on 1% agarose gels in TAE buffer (1.6 M Tris, 0.8 M sodium acetate, 40 mM EDTA-Na_2, pH 7.2), using a mini-gel apparatus. The samples were electrophoresed at 100 V for 30 to 60 minutes, and the gels stained with 5 mg/ml ethidium bromide (Sigma Chemical Company, St Louis, MO, USA) for 5 minutes and photographed under ultra violet light (245 nm).

4.2.11 Digestion of plasmid DNA with restriction enzymes

DNA digestion was performed at 37°C, using the enzyme conditions specified by the suppliers. Digestions were carried out in a final reaction volume of 20 µl for less than 5 µg of DNA, or larger volumes for greater quantities of DNA. Restriction digests were incubated at the temperatures specified for 2 hours or overnight when more than 20 µg of DNA were digested. Complete restriction of the DNA was checked by running a fraction of the restriction digest with 1 x glycerol loading buffer (50% glycerol, 1% bromophenol blue, 1% xylene cyanol in 1 x TAE buffer) on 1% or 1.5% agarose gels. When digests required more than one enzyme, the enzyme that required the lower salt concentration was used first, and the salt concentration adjusted to the conditions required for digestion with the second restriction enzyme.

4.2.12 Isolation of DNA fragments from agarose gels

Digested DNA samples were electrophoresed on a 0.8 % agarose gel at 90 V for 1 hour. The gel was stained with ethidium bromide, the fragment localised under UV light (245 nm) and cut out of the gel. The gel slice was placed into a 0.5 ml Eppendorf tube that had been pierced with a 21 G hypodermic needle at the base and plugged with glass wool. This tube was placed inside a 1.5 ml Eppendorf tube and centrifuged at 6,500 x *g* for 10 minutes. The eluate collected in the 1.5 ml Eppendorf tube was stored at 4°C. To the gel slice was added 50 µl TE buffer and incubated at 4°C for 1 hour. The Eppendorf assembly containing the gel slice was centrifuged at 6,500 x *g* for 10 minutes and the eluate pooled with that previously collected. This step was repeated, and the pooled eluate samples were extracted twice with an equal volume of phenol : chloroform (1:1) followed by a chloroform extraction. The DNA fragments were precipitated in 2.5 volumes 100% ethanol in the presence of 0.3 M

sodium acetate, pH 5.2 and centrifuged at 12,500 x *g* for 15 minutes. The pellet was washed in 70 % ethanol, dried in a vacuum centrifuge and resuspended in 15 µl TE buffer. A 1 µl aliquot of the isolated fragment was electrophoresed on a 1% agarose gel to visualise the integrity of the DNA fragment.

4.2.13 Ligations

All ligation reactions were carried out in a final reaction volume of 25 µl with approximately 50 - 500 ng of DNA. Several ligations with ratios of vector : insert ranging from 1:2 to 1:10 were performed for each ligation experiment. DNA ligations were carried out using DNA of various vector : insert ratios in the presence of 1 x ligase buffer (0.5 M Tris-HCl, pH 7.4, 0.1 M $MgCl_2$, 0.2 M ATP, 50 mg/ml BSA), 1 mM ATP and 1-2 units T_4 DNA ligase and incubated at 14°C for 3 hours. Ligation mixes were then transformed into bacterial cells as described in section 4.2.8.

4.2.14 DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method using a dideoxynucleotide sequencing kit (Pharmacia-LKB Biotech Inc., Uppsala, Sweden). Approximately 3 µg of plasmid DNA was resuspended in 5 µl H_2O, mixed with 10 ng (1 µl) M13 universal primer and denatured with 1 µl 1 M NaOH for 10 minutes at 37°C. To neutralise the denatured solution, 1 µl 1 M HCl was added followed by the addition of 2 µl 5 x sequencing buffer (200 mM Tris HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). To ensure primer/template annealing, the reaction was incubated at 37°C for 5 minutes. To the tube was added 1 µl 0.1M DTT, 2 µl labelling mix (a 1 in 5 dilution of 7.5 mM each dGTP, dCTP and dTTP) 5 mCi α-^{35}S dATP and 2 µl T7 DNA polymerase (diluted 1 in 8) and incubated at room temperature for 5 minutes. Aliquots of 4.5 µl were divided into 4 Eppendorf tubes containing 2.5 µl of one of the termination mixes (see Table 1). The tubes were incubated at 37°C for 5 minutes and the reaction terminated by the addition of 5 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were heated at 80°C for 2 minutes prior to loading onto a sequencing gel prepared as described in section 4.2.15

Table 4.1:

Composition of termination mixes for dideoxy sequencing

ddG termination mix	ddA termination mix	ddT termination mix	ddC termination mix
80 μM dGTP	80 μM dGTP	80 μM dGTP	80 μM dGTP
80 μM dATP	80 μM dATP	80 μM dATP	80 μM dATP
80 μM dCTP	80 μM dCTP	80 μM dCTP	80 μM dCTP
80 μM dTTP	80 μM dTTP	80 μM dTTP	80 μM dTTP
8 μM ddGTP	8 μM ddATP	8 μM ddTTP	8 μM ddCTP
50 mM NaCl	50 mM NaCl	50 mM NaCl	50 mM NaCl

4.2.15 Polyacrylamide gel electrophoresis

Sequencing gels containing 6% polyacrylamide were prepared by mixing 80 ml Sequagel-6™ monomer solution with 20 ml Sequagel-6™ buffer (National Diagnostics, Atlanta, GA, USA). To allow polymerisation, 0.64 ml 10% ammonium persulphate (Sigma Chemical Company, St. Louis, MO, USA) was mixed with the acrylamide solution and the gel was poured between two glass plates 0.4 mm apart. Gels were electrophoresed in a vertical gel apparatus in TBE buffer (50 mM Tris-HCl; 50 mM boric acid; 1 mM EDTA-Na_2) at 65 watts for 1 to 2 hours, fixed in 12% acetic acid, 20% ethanol for 10 minutes and dried for 30 minutes using a gel dryer (Biorad Laboratories Inc., Richmond, CA, USA) before exposure to phosphorimager screens.

4.2.16 Analysis of gels

All gels were scanned using a phosphorimager model # 425E (Molecular Dynamics, USA) and Image Quant version 3.2 software (Molecular Dynamics, USA). Sequencing gels were read manually. RNase protection assay gels were quantitated by measuring the intensity (by measuring the integrated volume) of each band, and expression of IGF-I and IGFBP-3 mRNA was normalised by expressing the intensity of these bands as a percentage of the intensity obtained for hybridisation of the same RNA aliquot to a 18S ribosomal RNA probe.

4.2.17 Subcloning of porcine IGFBP-3 DNA fragments into plasmid vectors

The strategy used for subcloning the porcine IGFBP-3 gene is shown in Figure 4.5. A 595 base pair fragment encoding the first 3 exons of porcine IGFBP-3 was originally subcloned into the Eco R1/Sma I sites of the multiple cloning region of pBluescript® II SK+ (Shimasaki et al., 1990). Analysis of the DNA sequence revealed several unique restriction sites suitable for subcloning. The original plasmid, pPBP3ES, was digested with either Eco R1 and Pst 1 (Figure 4.5a), Pst 1 and Sal I (Figure 4.5b) or Sal I and Sma I (Figure 4.5c) to yield 3 separate fragments that coded for either exon 1, part of exons 1 and 2 or part of exons 2 and 3 of porcine IGFBP-3. These fragments were isolated from agarose gels as described in section 4.2.12 and ligated unidirectionally into the multiple cloning region of pBluescript® II SK+ as described in section 4.2.13. The ligation mixes were transformed into competent DH5α bacteria and plated onto agar plates containing 100 mg/ml ampicillin. Following incubation at 37°C overnight, ampicillin resistant colonies were selected and plasmid DNA was prepared by the small scale isolation method as described in section 4.2.6. Successful ligation of the IGFBP-3 fragments into pBluescript® II SK+ was verified by digestion with the appropriate restriction enzymes and electrophoresis on 1% agarose gels. The DNA sequence was verified by DNA sequence analysis as described in section 4.2.14.

4.2.18 RNA isolation

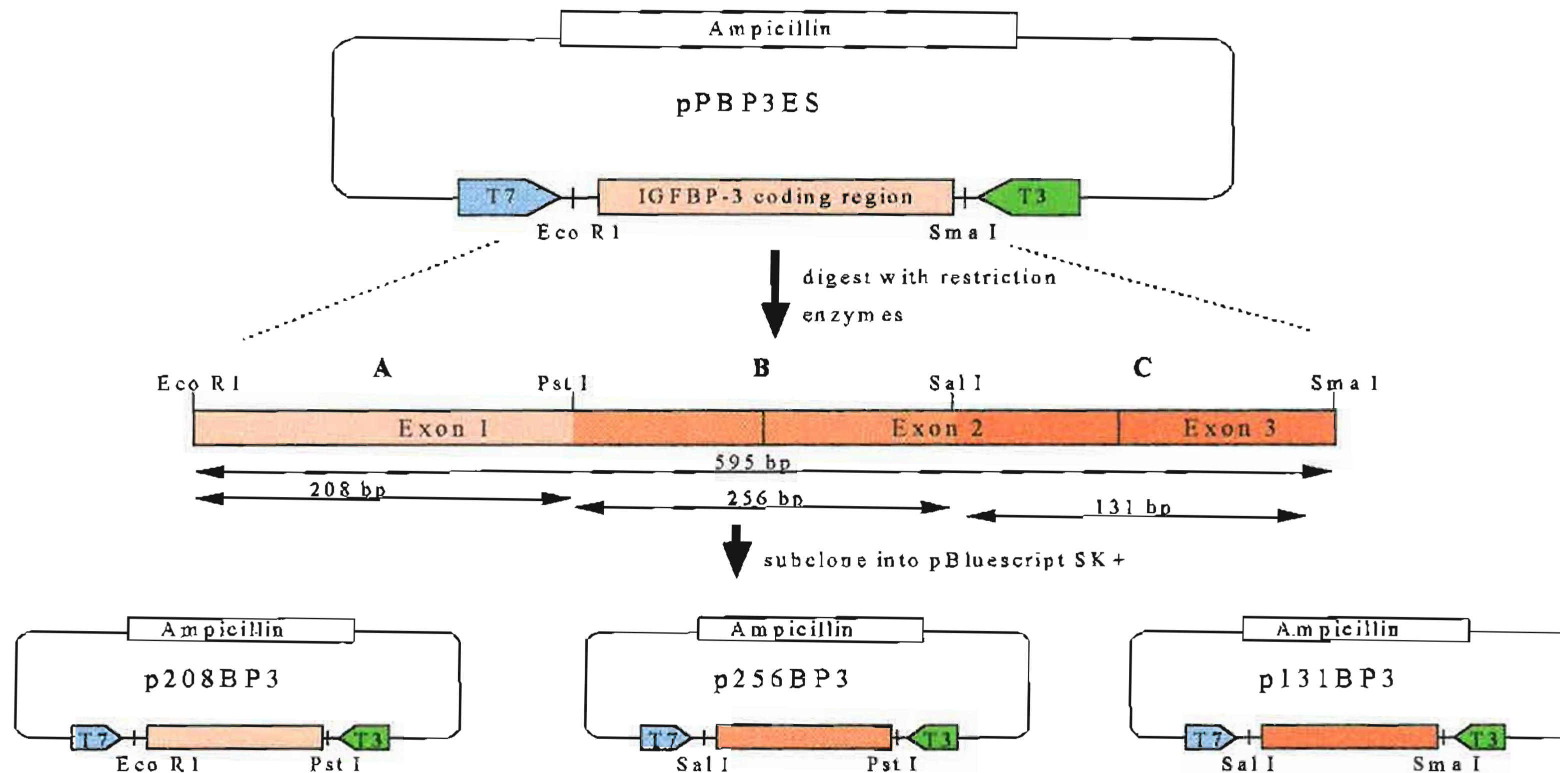
Total RNA was extracted from livers obtained from pigs treated with pGH. To 1 ml RNAzol™B (Tel-Test Inc., Friendswood, Texas, USA) was added approximately 2 grams of frozen tissue, and homogenised using an ultraturex homogeniser. To the homogenised sample was added 2 ml RNAzol™B and 0.4 ml chloroform. The samples were vigorously shaken for 10 seconds, aliquoted into sterile 1.5 ml Eppendorf tubes and centrifuged at 12,500 x *g* for 15 minutes at room temperature. Approximately 0.7 ml of the upper aqueous phase was removed to a fresh tube containing 0.5 ml RNAzol™B and 0.15 ml chloroform. The tubes were vigorously shaken for 10 seconds and centrifuged at 12,500 x *g* for 15 minutes at room temperature. Approximately 0.7 ml of the upper aqueous phase was removed to a fresh tube and an equal volume isopropanol added. The RNA was allowed to precipitate at 4°C for 1 hour and pelleted by centrifugation at 12,500 x *g* for 15 minutes at room temperature and the resultant pellet washed with 0.5 ml 4 M LiCl followed by a wash with 0.5 ml 70% ethanol.

The pellet was briefly air dried and resuspended in 100 µl DEP-C treated sterile glass distilled water (DEP-C H_2O). The pellet was resuspended at 4°C for 2 hours and if necessary, heated at 65°C for 5 minutes. The integrity of the RNA was established by 1% agarose gel electrophoresis and visualisation of ethidium bromide stained gels under UV light. RNA was quantitated by measuring the absorbance of a diluted RNA sample at 260 nm using a spectrophotometer.

4.2.19 Generation of radioactive DNA molecular weight markers

DNA molecular weight markers BMVIII (Boehringer Manneheim Biochemicals, Indianapolis, USA) were end labelled using a DNA 5' end labelling kit (Boehringer Manneheim Biochemicals, Indianapolis, USA). Briefly, 250 ng of marker DNA was incubated with 7 µl buffer B (10 mM Tris-HCl; 0.1 mM EDTA pH 8.2), 2 µl buffer D (0.5 M imidazole-HCl; 100 mM $MgCl_2$; 1 mM EDTA; 50 mM DTT; 1 mM spermidine; 3 mM ADP, pH 6.6), 50 µCi γ-^{32}P dATP and 8 units T_4 polynucleotide kinase for 30 minutes at 37°C. The reaction was terminated by the addition of 80 µl H_2O and incubation at 65°C for 10 minutes. Labelled markers were stored at -80°C for up to 4 weeks.

Figure 4.5: **Subcloning of porcine IGFBP-3**



Three separate restriction digests were carried out using pPBP3ES. To yield a fragment containing only exon 1 (see ▭) pPBP3ES was digested with Eco R1 and Pst I (A); to yield a fragment containing the 3' end of exon 1 and the 5' end of exon 2 (see ▭) pPBP3ES was digested with Pst I and Sal I (B); and to yield a fragment containing the remainder of exon 2 and the 5' end of exon 3 (see ▭) pPBP3ES was digested with Sal I and Sma I (C). These digests generated fragments that were 208 base pairs, 256 base pairs and 131 base pairs in length respectively. These fragments were isolated as described in the text and subcloned into pBluescript® II SK+.

4.2.20 Generation of high specific activity IGF-I class 1 or IGF-I class 2 riboprobes

To generate antisense probes for detection of IGF-I *class 1* or *class 2* mRNA, 10 µg pRP1 and pRP2 were linearised by overnight digestion with 10 units Bam H1. Completion of the digestion was visualised on ethidium bromide stained 1% agarose gels and the DNA purified by phenol : chloroform extraction and precipitation with 2.5 volumes of 100 % ethanol in the presence of 0.3 M sodium acetate pH 5.2. The DNA templates were resuspended in TE buffer at a concentration of 100 ng/µl. Riboprobes were generated using a T7/T3 transcription kit (Promega Corporation, Madison, WI, USA). The transcription reaction was initiated by adding, in order: 2 µl 5x transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl) 1 µl 100 mM DTT, 0.5 µl RNasin, 2 µl ATP, CTP, GTP mix (7.5 mM each) 1.2 µl 100 mM UTP, 0.5 µl linearised template DNA, 25 µCi α-^{32}P UTP and 10 units T7 RNA polymerase and incubated at 37°C for 60 minutes. The DNA template was degraded by the addition of 0.5 µl RNase free DNase and incubation at 37°C for 15 minutes. To the reaction was added 80 µl DEP-C H_2O and 42 µl phenol : chloroform : iosamyl alcohol (25:24:1) followed by thorough mixing and centrifugation at 12,500 x *g* for 5 minutes. The upper aqueous phase (90 µl) was removed to a fresh tube and the riboprobe precipitated by the addition of 10 µl 10 mg/ml tRNA (Sigma Chemical Company, St Louis, MO, USA), 4 µl 5M NaCl and 250 µl ethanol and centrifuged at 12,500 x *g* for 15 minutes. The RNA pellet was washed with 500 µl 70 % ethanol and resuspended in 40 µl DEP-C H_2O. To determine percent of incorporation of the radioactive nucleotide, 1 µl of the riboprobe mix was aliquoted into 2 ml scintillant (Beckmann Incorporated, Fullerton, CA, USA) immediately after the completion of the transcription reaction and after the RNA pellet had been precipitated with ethanol and resuspended in DEP-C H_2O. Radioactive emission was measured using a β counter and the activity of the probe adjusted to 5×10^5 cpm/µl.

4.2.21 Generation of high specific activity IGFBP-3 riboprobes

To generate antisense probes for the detection of porcine IGFBP-3 mRNA, 10 μg pBP3ES, p256BP3, p208BP3 or p131BP3 were linearised by overnight digestion with 10 units EcoR1, Sal I, Pst 1and Sal I respectively. Completion of digestion was visualised on ethidium bromide stained 1% agarose gels and the linearised plasmid DNA purified by phenol : chloroform extraction and precipitation with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate pH 5.2. The DNA template was resuspended in TE buffer at a concentration of 100 ng/μl. Generation of riboprobes for porcine IGFBP-3 was identical as described in section 4.4.20 except that T3 RNA polymerase was used when transcribing riboprobes using plasmids pBP3ES, p256BP3 and p131BP3.

4.2.22 Generation of low specific activity 18S riboprobes

To generate a low specific activity probe, excess cold UTP was required. To a 1.5 ml screw cap Eppendorf tube was added in order: 4 μl 5x transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl) 2 μl 100 mM DTT, 0.5 μl RNasin, 12 μl ATP, CTP, GTP, UTP mix (7.5 mM each), 1 μl (100 ng) pT7 18S RNA, 1 μCi α-^{32}P UTP and 20 units T7 RNA polymerase followed by incubation at 37°C for 60 minutes. The DNA template was degraded by the addition of 0.5 μl RNase free DNase and incubation at 37°C for 15 minutes. To the reaction was added 80 μl DEP-C H_2O and 42 μl phenol : chloroform : iosamyl alcohol (25:24:1) followed by thorough mixing and centrifugation at 12,500 x *g* for 5 minutes. The upper aqueous phase (90 μl) was removed to a fresh tube and the riboprobe precipitated by the addition of 10 μl 10 mg/ml tRNA, 4 μl 5M NaCl and 250 μl of 100 % ethanol and centrifuged at 12,500 x *g* for 15 minutes. The RNA pellet was washed with 500 μl 70 % ethanol and resuspended in 30 μl DEP-C H_2O. To determine percent of incorporation of the radioactive nucleotide, 1 μl of the riboprobe mix was aliquoted into 2 ml scintillant immediately after the completion of the transcription reaction and after the RNA pellet had been precipitated with ethanol and resuspended in DEP-C H_2O. Radioactive emission was measured using a β counter and the activity of the probe adjusted to 5 x 10^4 cpm/μl.

4.2.23 RNase protection assay

To optimise RNA loading for detection of the various mRNA transcripts, the RNase protection assay method was performed with varying quantities of RNA ranging from 0 to 100 μg. The RNA was hybridised with different riboprobes, and concentration curves established to determine the optimum amounts of RNA for the detection of IGF-I *class 1* and IGFBP-3 mRNA and 18S ribosomal RNA. RNA samples were analysed in duplicate. The composition of the hybridisation reactions is shown in Table 4.2.

Table 4.2:

Composition of hybridisation reactions for RNase protection assays

Pig sample	RNA	formamide	hyb. buffer[1]	Probe	DEP-C H_2O
1. Sample 1	*x* μl	20 μl	8 μl	1 μl	make to 40 μl
2. Sample 1	"	"	"	"	"
3. Control	25 μg tRNA	"	"	"	"
4. Control	"	"	"	"	"

[1] *Hybridisation buffer: 125 mM PIPES free acid pH 6.8, 2 M NaCl, 5 mM EDTA pH 8.0*

The tubes were thoroughly mixed, incubated at 85°C for 10 minutes followed by incubation at 45°C overnight. To each tube , except tube #4 in the above example was added 300 μl RNase digestion buffer (10 mM Tri-HCl, pH 7.6; 5 mM EDTA; 300 mM NaCl) containing 40 μg/ml RNase A and 2 μg/ml RNase T1. To the last tube in each set of reactions was added 300 μl RNase digestion buffer only to allow the detection of the full length probe. The tubes were incubated at 37°C for 60 minutes, except when the pRP1 riboprobe was used when incubation was carried out at room temperature for 60 minutes. Following digestion of single stranded RNA, proteins were denatured by the addition of 200 μl 10% SDS and 5 μl 20 mg/ml Proteinase K and incubation at 37°C for 15 minutes. Proteins were removed from the RNA hybrids by extraction with 400 μl phenol : chloroform : isoamyl alcohol (25:24:1) and centrifugation at 12,500 x *g* for 5 minutes. The upper aqueous phase (400 μl) was removed to a fresh tube containing 800 μl of 100 % RNase free ethanol and the RNA precipitated by centrifugation at 12,500 x *g* for 15 minutes. The RNA pellet was resuspended at 65°C for 5 minutes in 4 μl loading dye (80% formamide; 10 mM EDTA; 1 mg/ml xylene cyanol FF; 1 mg/ml bromophenol blue), vortexed, heated at 95°C for 3 minutes and loaded onto a polyacrylamide gel prepared as described in section 4.2.15.

4.3 Results

4.3.1 The porcine IGF-I riboprobes

The plasmids pRP1 and pRP2 were transformed into competent DH5α cells and plasmid DNA was prepared for verification of the DNA sequence. The DNA sequence and accompanying autoradiographs for pRP1 and pRP2 are shown in Figures 4.6 and 4.7 respectively.

4.3.2 Construction of the porcine IGFBP-3 riboprobe

pPBP3ES contains a 595 base pair insert coding for the first 3 exons of porcine IGFBP-3. Digestion of this plasmid with Eco R1 and Sma I released the fragment from its host vector, pBluescript® II SK+ (Figure 4.8). Digesting pPBP3ES with Eco RI (Figure 4.8, lane 3), purifying the linearised template and initiating transcription from the T3 RNA promoter contained within pBluescript® II SK+ generated a antisense RNA probe complementary to the first three exons of porcine IGFBP-3 mRNA. This riboprobe was used in a RNase protection assay and hybridised to porcine liver RNA. Analysis of the results revealed that the 665 nucleotide riboprobe generated was of the expected size, however no protected fragments could be detected at the expected size of 595 bases and multiple bands were identified at approximately 250 and 238 bases (Figure 4.9). Investigation of the DNA sequence revealed a region rich in GC residues at the 3' end of exon 1. The generation of two protected fragments 250 and 238 bases in length is consistent with a RNase sensitive region at the 3' end of exon 1. This required the subcloning of the IGFBP-3 fragment in order to remove the GC rich region from the riboprobe. The strategy employed is outlined in Figure 4.5 and the results are presented in Figure 4.10. Digestion with Eco R1 and Pst I produced a 208 base pair, exon 1 specific fragment that encompassed the GC rich region (Figure 4.10a lane 2). Digestion with Pst I and Sal I produced a 256 base pair fragment encoding the 3' end of exon 1 and the 5' end of exon 2 (Figure 4.10a lane 3), and digestion with Sal I and Sma I produced a 131 base pair fragment that encoded the remainder of exon 2 and the 5' end of exon 3 (Figure 4.10a lane 4). These fragments were subcloned into pBluescript® II SK+ and linearised with Eco R1, Pst I and Sal I respectively (Figure 4.10b) to be used as linearised templates for the production of antisense riboprobes for different regions of IGFBP-3.

Figure 4.6: **DNA sequence of plasmid pRP1**

A:

oligo 278 →

BamH1

Ser Ser Leu Pro Thr Gln Leu Phe Lys Cys Cys Phe
5' CGCATAGGATCCTC AGC AGT CTT CCA ACC CAA TTA TTT AAG TGC TGC TTT
3' AA TTC ACG ACG AAA

exon 1 & 3 junction

Cys Asp Phe Leu Lys | Val Lys Met His Ile Thr Ser Ser Ser His Leu Phe
TGT GAT TTC TTG AAG | GTA AAG ATG CAC ATC ACA TCC TCT TCG CAT CTC TTC
ACA CTA AAG AAC TTC |CAT TTC TAC GTG TAG TGT AGG ACA AGC GTA GAG AAG

Tyr Leu Ala Leu Cys |Leu Leu Ser Phe Thr Ser Ser Ala Thr Ala **Gly** **Pro**
TAC TTG GCC CTG TGC TTG CTC TCC TTC ACC AGC TCT GCC ACG GCT GGA CCT
ATG AAC CGG GAC ACG AAC GAG AGG AAG TGG TCG AGA CGG TGC GAC TGG GGA

Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe Val
GAG ACC CTC TGT GGG GCT GAG CTG GTG GAC GCT CTT CAG TTC GTG
CTC TGG GAG ACA CCC CGA CTC GAC CAC CTG CGA GAA GTC AAG CAC

Cys Gly Asp Arg Gly
TGC GGA GAC AGG GGC T 3'
ACG CCT CTG TCC CCG A CTTAAGCTATAGTTCG 5'

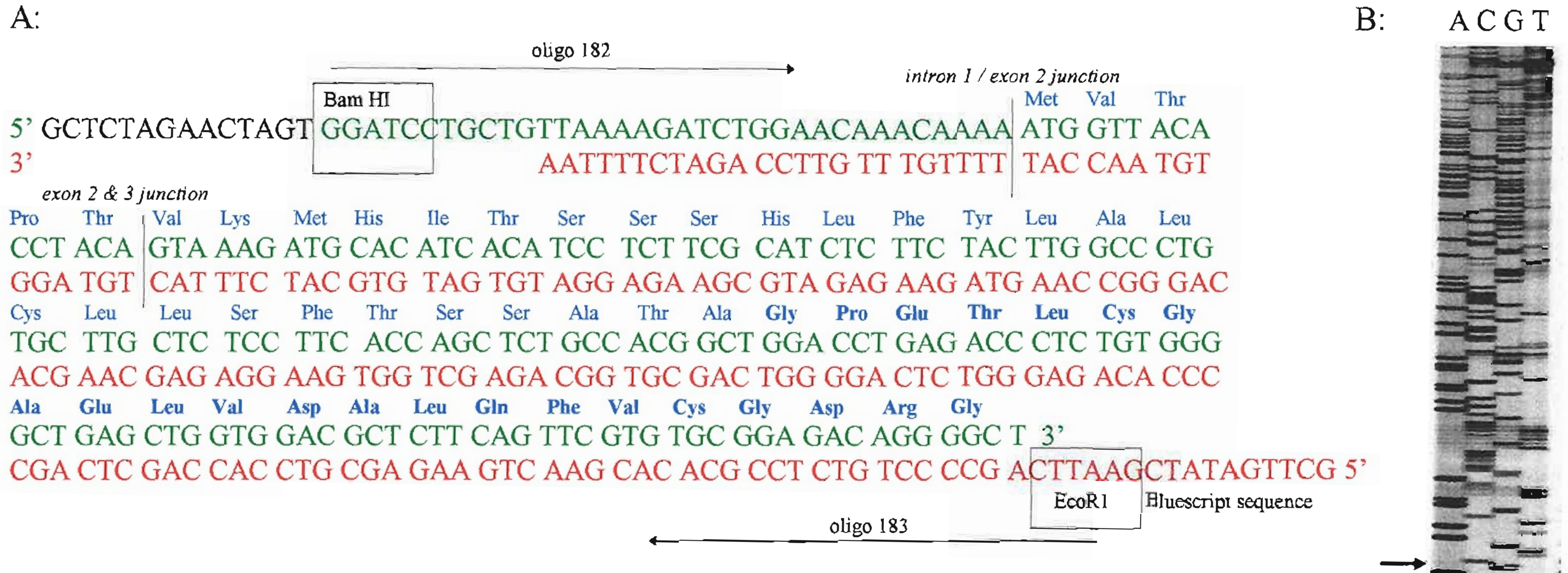
EcoR1 Bluescript sequence

← oligo 183

B:

A: Amino acids coding for the IGF-I class 1 leader and exon 3 sequence are indicated in blue, with the amino acids coding for the mature IGF-I protein in bold. The published DNA sequence is indicated in green, and the sequence read from the accompanying autoradiograph (B) is represented in red. The arrow indicates the 5' end of the sequence read. The primer regions (oligo 278 and 183) used in PCR amplification of the insert and restriction sites for unidirectional cloning into pBluescript® II SK+ are also indicated.

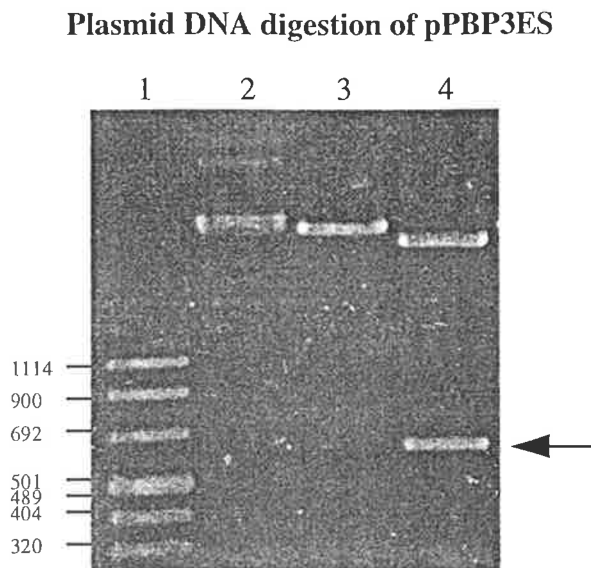
Figure 4.7: **DNA sequence of plasmid pRP2.**



A: Amino acids coding for the IGF-I class 2 leader and exon 3 sequence are indicated in blue, with the amino acids coding for the mature IGF-I protein in bold. The published DNA sequence is indicated in green, and the sequence read from the accompanying autoradiograph (B) is represented in red. The arrow indicates the 5' end of the sequence read. The primer regions (oligo 182 and 183) used in PCR amplification of the insert and restriction sites for unidirectional cloning into pBluescript® II SK+ are also indicated.

Figure 4.8:

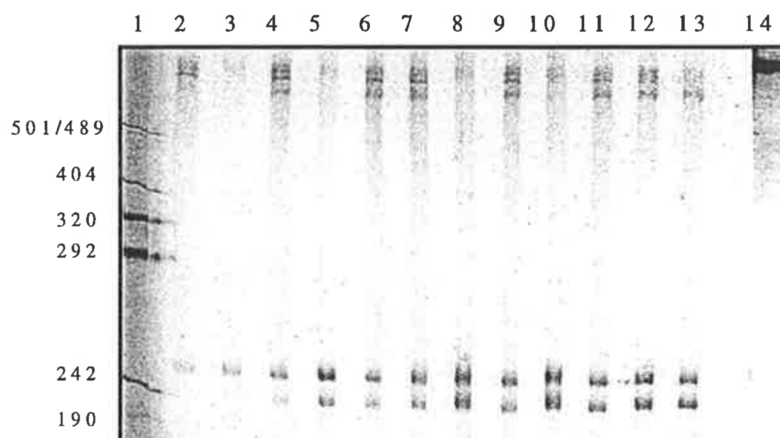
Plasmid DNA digestion of pPBP3ES



Lane 1: DNA molecular weight markers whose sizes are indicated in bases; Lane 2: intact pPBP3ES; Lane 3: pPB3ES linearised by digestion with Eco R1; Lane 4: pPBP3ES digested with Eco R1 and Sma I to release a 595 base pair fragment (indicated by arrow) coding for the first three exons of porcine IGFBP-3.

Figure 4.9:

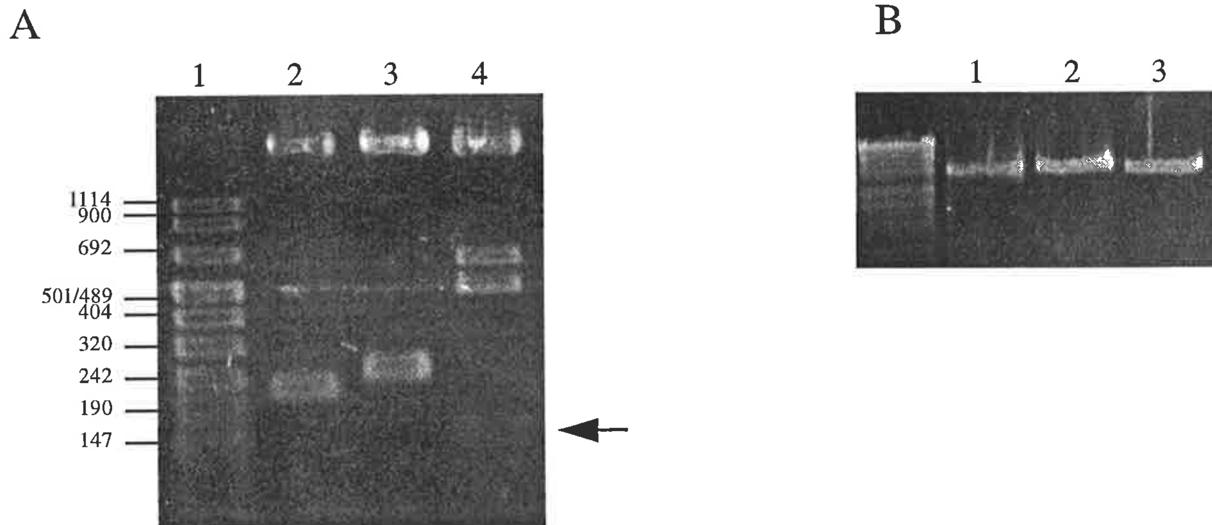
RNase protection assay of porcine liver RNA hybridised to a 665 nucleotide riboprobe complementary to the first 3 exons of porcine IGFBP-3.



Lane 1: Molecular weight markers whose sizes are indicated in base. Two protected fragments corresponding to 250 and 238 bases were identified when porcine liver RNA was hybridised with the 665 nucleotide riboprobe (lane 14) in increasing amounts, 10 µg (lanes 2 & 3), 20 µg (lanes 4 & 5), 30 µg (lanes 6 & 7), 40 µg (lanes 8 & 9), 50 µg (lanes 10 & 11) and 60 µg (lanes 12 & 13).

Figure 4.10:

Subcloning of the porcine IGFBP-3 gene



(A) Lane 1: DNA molecular weight markers whose sizes are indicated; Lane 2: pPBP3ES digested with Eco R1 and Pst I to generate a 208 base pair fragment coding for exon 1 sequence. Lane 3: pPBP3ES digested with Pst I and Sal I to generate a 256 base pair fragment coding for exon 1 and exon 2 sequence. Lane 4: pPBP3ES digested with Sal I and Sma I to generate a 131 base pair fragment (indicated by arrow) coding for exon 3 sequence. Two bands between 500 and 700 base pairs were also generated. They may represent DNA digestion at additional sites in the plasmid sequence, although pBluescript® II SK+ has no other restriction sites for Sal I or Sma I. The small DNA fragments were isolated from the gel and subcloned into pBluescript® II SK+ as described in the text.

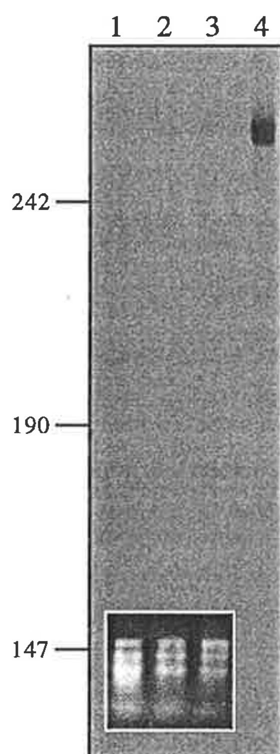
(B) Preparation of antisense transcription templates. Lane 1: p208BP3 linearised with Eco R1; Lane 2: p256BP3 linearised with Pst I; Lane 3: p131BP3 linearised with Sal I. Transcription templates were isolated and purified as described in the text.

A exon 1 specific riboprobe was generated by transcribing from the T3 RNA promoter of Eco RI linearised p208BP3. This 278 nucleotide riboprobe was of the expected size but failed to resolve any protected fragments when hybridised with porcine liver RNA (Figure 4.11).

The exon 1/2 specific riboprobe was generated by transcribing from the T7 RNA promoter of Pst I linearised p256BP3. The resultant protection assay is shown in Figure 4.12. The expected length of this riboprobe was 289 nucleotides, however three species of riboprobes were generated. A major transcript of 265 nucleotides was accompanied by two additional transcripts 281 and 289 nucleotides in length. When this probe was hybridised with porcine liver RNA in a RNase protection assay, one protected fragment 158 bases in length was generated. This does not agree with the expected size of 256 bases.

Figure 4.11:

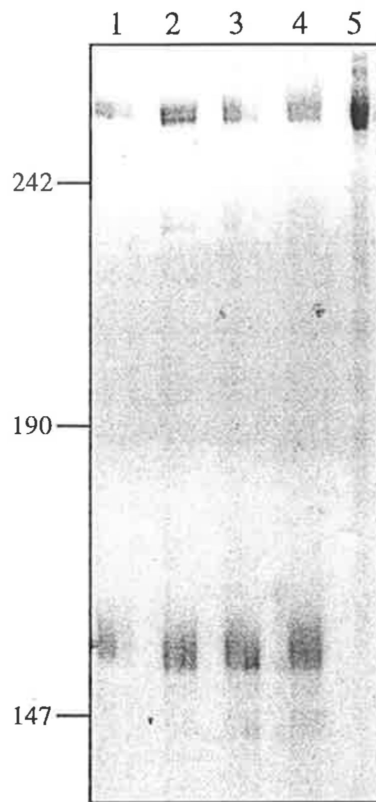
Detection of IGFBP-3 gene expression in porcine liver



Porcine liver RNA was hybridised with a riboprobe generated from p208BP3. Hybridisation of 10, 20 and 40 μg liver RNA (lanes 1, 2 & 3 respectively) with an exon 1 specific riboprobe failed to generate any protected fragments. Lane 4 represents the full length probe 278 nucleotides in length. RNA was of good quality when analysed by agarose gel electrophoresis (insert). Molecular marker sizes are indicated in bases.

Figure 4.12:

Detection of IGFBP-3 gene expression in porcine liver.

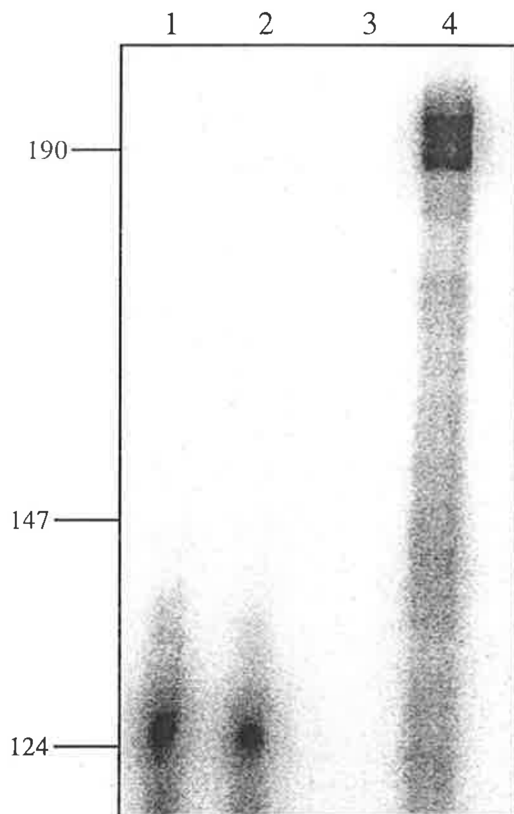


Porcine liver RNA was hybridised with a riboprobe generated from p256BP3. Hybridisation of 5, 10, 15 and 20 μg liver RNA (lanes 1, 2 3 and 4 respectively) with an exon 1/2 specific riboprobe produced a fragment 158 bases in length. This is smaller than the expected size of 256 bases. Molecular weight marker sizes are indicated in bases. Lane 5 shows the three species of probes generated.

The exon 2/3 specific riboprobe was generated by transcribing from the T3 RNA promoter of Sal I linearised p131BP3. The resultant protection assay is shown in Figure 4.13. As expected, the 192 nucleotide riboprobe protected a fragment 139 bases in length when hybridised with porcine liver RNA. These results indicated that p131BP3 was suitable for generating porcine IGFBP-3 specific riboprobes in RNase protection assays to detect expression of IGFBP-3 mRNA. The DNA sequence of p131BP3 and the accompanying autoradiograph are shown in Figure 4.14.

Figure 4.13:

Detection of IGFBP-3 gene expression in porcine liver.



Porcine liver RNA was hybridised with a riboprobe generated from p131BP3. Hybridisation of 50 μg liver RNA (lanes 1 & 2) with an exon 2/3 specific probe generated a 139 nucleotide protected fragment that corresponded to the expected size. Lane 3 represents complete digestion of probe with RNase and lane 4 represents the full length probe 192 nucleotides in length. Molecular weight marker sizes are indicated in bases.

Figure 4.14: **DNA sequence of plasmid p131BP3**

A:

Bluescript.... Sal I site

Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser

5' TCGAG GTC GAC TAC GAG TCT CAG AGC ACG GAC ACC CAG AAC TTC TCC

Exon 2 & 3 junction

Ser Glu Ser Lys Arg Glu Thr Glu Tyr | Gly Pro Cys Arg Arg Glu

TCT GAG TCC AAG CGC GAG ACG GAA TAC | GGG CCT TGC CGC CGG GAA

Met Glu Asp Thr Leu Asp His Leu Lys Phe Leu Asn Met Leu Ser

ATG GAG GAC ACG CTG AAC CAC CTC AAG TTC CTG AAC ATG CTC AGC

Pro Arg

CCC CGG G GATCCACTAGT 3'

Sma I site Bluescript sequence

B:

A: Amino acids coding for part of exons 2 and 3 of porcine IGFBP-3 are indicated in blue. The DNA sequence obtained from DNA sequence analysis (B) is indicated in red and corresponds with the published sequence of IGFBP-3. The arrow indicates the 5' end of the sequence read.

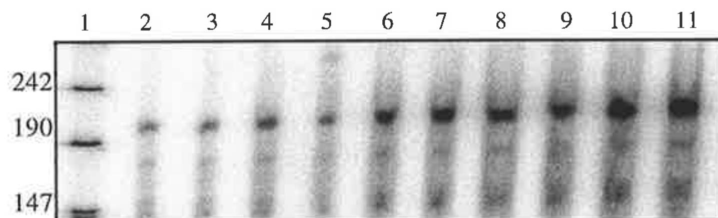
4.3.3 Optimisation of quantity of RNA to be used in RNase protection assays

To determine the optimum amount of RNA to be used in RNase protection assays for detection of IGF-I and IGFBP-3 mRNA and 18S ribosomal RNA, the riboprobe vectors were linearised by digestion with the relevant restriction enzymes and riboprobes was prepared by initiating transcription from the T7 or T3 RNA promoters. A RNA concentration curve was established by hybridising 5 x 10^5 cpm of antisense IGF-I *class 1* or IGFBP-3 RNA probe with either 20, 40, 60, 80, or 100 µg of porcine liver RNA. Due to the abundance of ribosomal RNA, a concentration curve using much less RNA (2, 5, 10, 20 or 40 µg of porcine liver RNA) was established for detection of 18S ribosomal RNA. Detection of IGF-I *class 1* expression using a riboprobe generated from pRP1 showed a linear response with increasing RNA concentration between 40 and 100 µg of porcine liver RNA (Figure 4.15).

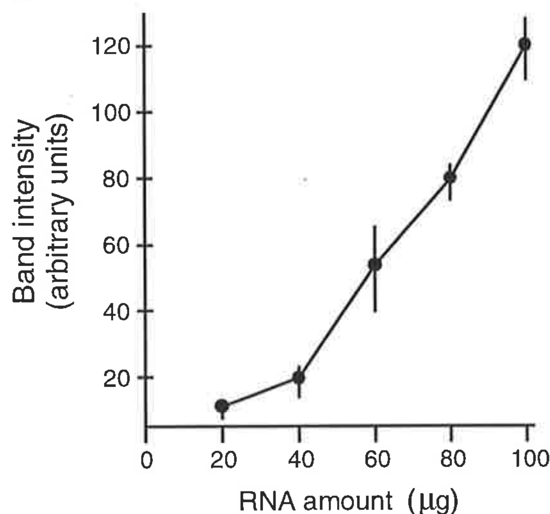
Figure 4.15:

Detection of IGF-I *class 1* mRNA expression in porcine liver with increasing amounts of total RNA

A



B



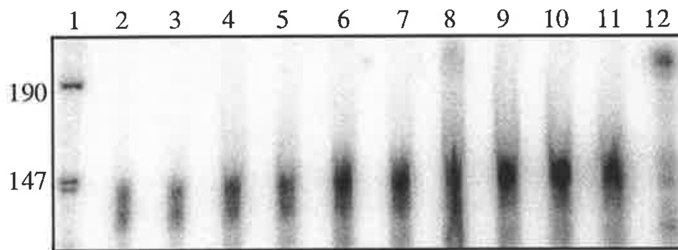
(A) Lane 1 represents molecular weight markers, whose sizes (in bases) are shown. Lanes 2 through to 11 are the results of hybridising duplicate samples of porcine liver RNA (20, 40, 60, 80, and 100 µg respectively) with a riboprobe specific for IGF-I class 1 mRNA. The intensity of two bands corresponding to 200 and 170 bases were quantitated for each lane and are graphically represented (B). Data are mean ± SEM, n = 2.

Detection of IGFBP-3 expression using a riboprobe generated from p131BP3 showed a linear response with increasing RNA between 20 and 60 μg of the same porcine liver RNA aliquot (Figure 4.16).

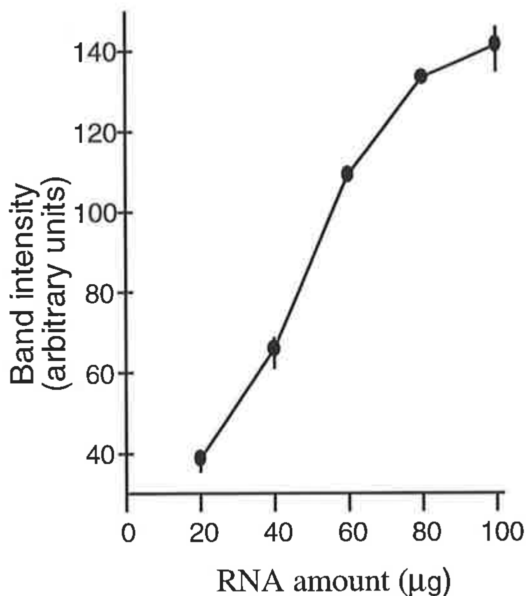
Figure 4.16:

Detection of IGFBP-3 mRNA expression in porcine liver with increasing amounts of total RNA

A



B

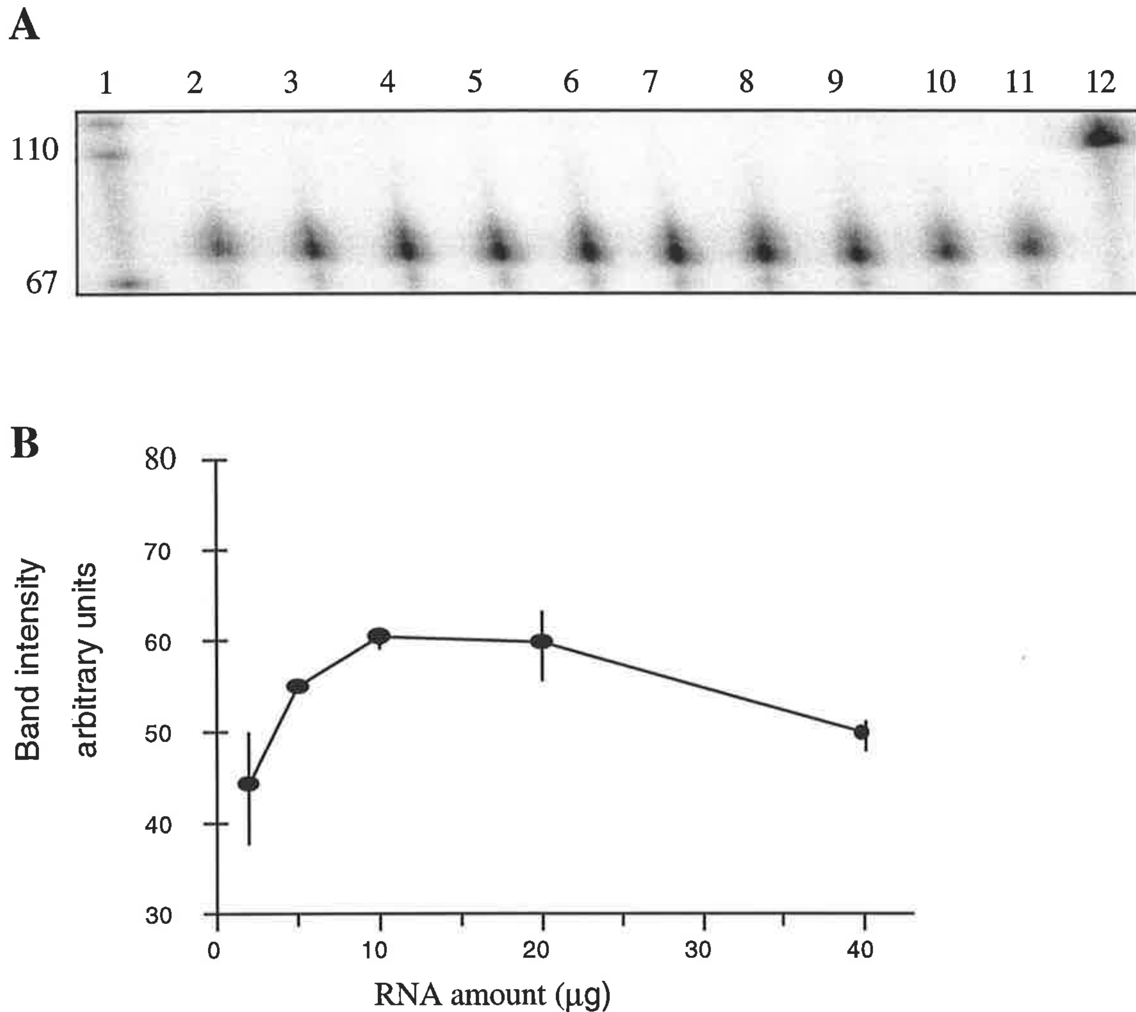


(A) Lane 1 represents molecular weight markers, whose sizes (in bases) are shown. Lanes 2 through to 11 are the results of hybridising duplicate samples of porcine liver RNA (20, 40, 60, 80, and 100 μg respectively) with a riboprobe specific for IGFBP-3 mRNA. Lane 12 represents the full length riboprobe. The intensity of a major band 139 bases in length was quantitated for each lane and is graphically represented (B). Data are mean ± SEM, n = 2.

Detection of 18S ribosomal RNA showed a linear response between 2 and 10 μg of porcine liver RNA, while increasing the amount of RNA beyond this resulted in a decrease in RNA hybridisation to a riboprobe generated from pT7 18S RNA (Figure 4.17).

Figure 4.17:

Detection of 18S ribosomal RNA expression in porcine liver with increasing amounts of total RNA



(A) Lane 1 represents molecular weight markers, whose sizes (in bases) are shown. Lanes 2 through to 11 are the results of hybridising duplicate samples of porcine liver RNA (2, 5, 10, 20, and 40 µg respectively) with a riboprobe specific for 18S RNA. Lane 12 represents the full length riboprobe. The intensity of a major band 80 bases in length was quantitated for each lane and is graphically represented (B). Data are means ± SEM, n = 2.

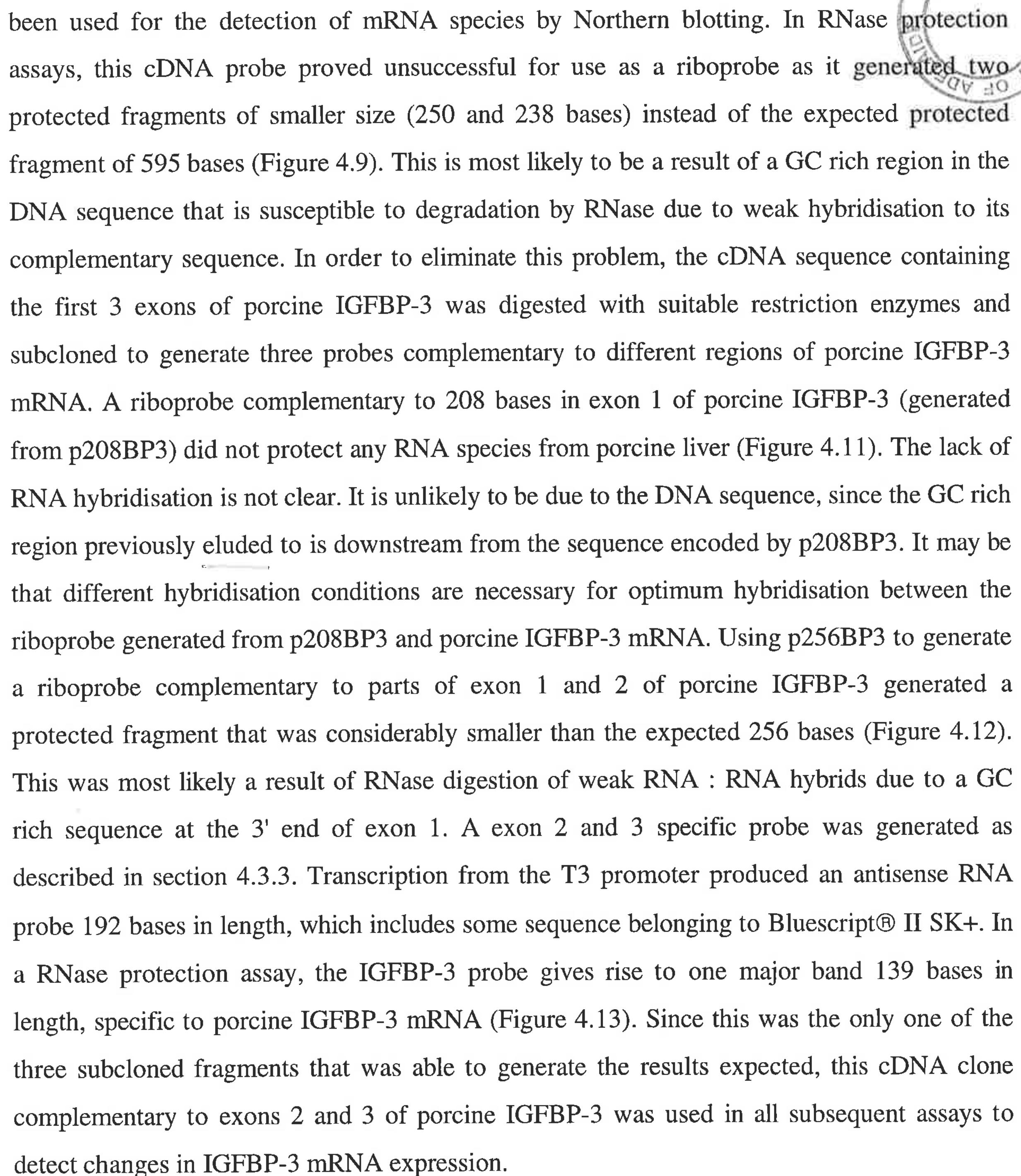
4.4 Discussion

The existence and alternate splicing of the two leader exons of the porcine IGF-I gene has been determined and their transcription from separate start sites investigated using riboprobes for the upstream regions of each of the leader exons (Weller *et al.*, 1993). The generation of these riboprobes specific for the two different precursor peptide mRNAs has made it possible to examine differential usage of leader exons at the mRNA level.

IGF-I *class 1* gene transcription is initiated upstream of leader exon 1 and is alternatively spliced to exon 3 which contains the remainder of the leader peptide sequence and the amino terminal of the mature protein (see Figure 1.3). The IGF-I *class 1* riboprobe (pRP1) was generated by PCR amplification of porcine liver RNA using oligonucleotides derived from the 5' end sequence of exon 1 and the 3' end sequence of exon 3 (see Figure 4.6). The resulting PCR fragment was cloned unidirectionally into Bluescript® II SK+ (Weller *et al.*, 1993). Transcription from the T7 promoter produces an antisense RNA 270 bases in length, which includes some sequence belonging to Bluescript® II SK+. In a RNase protection assay, the IGF-I *class 1* probe gives rise to two bands specific for IGF-I *class 1* mRNA. A full length protected band of 200 bases and a truncated band of 170 bases arising due to a temperature and RNase sensitive site within the sequence (Figure 4.15). A third, shorter band of 147 bases is also present, representing hybridisation to non IGF-I *class 1* transcripts comprising of exon 3 sequence only (Weller *et al.*, 1993).

IGF-I *class 2* gene transcription is initiated upstream of leader exon 2 and is spliced to exon 3 (see Figure 1.3). The IGF-I *class 2* riboprobe (pRP2) was generated by PCR amplification of porcine liver RNA using oligonucleotides derived from the 5' end sequence of exon 2 and the common 3' end sequence of exon 3 (see Figure 4.7). The resulting PCR fragment was cloned unidirectionally into Bluescript® II SK+ (Weller *et al.*, 1993). Transcription from the T7 promoter produces an antisense RNA 260 bases in length, which includes some sequence belonging to Bluescript® II SK+. In a RNase protection assay, the IGF-I *class 2* probe gives rise to one band 200 bases in length, specific for IGF-I *class 2* mRNA. A second band of 147 bases is also present, representing hybridisation to non IGF-I *class 2* transcripts comprising of exon 3 only (Weller *et al.*, 1993).

There is only one mRNA species coding for porcine IGFBP-3. It is 2.6 kb in length and contains 5 exons with the mature protein being encoded in the first 4 exons (Shimasaki *et al.*, 1990). A plasmid containing the first three exons of porcine IGFBP-3 was kindly donated by Dr. S. Shimasaki. This cDNA probe has so far only been cited in the literature as having



been used for the detection of mRNA species by Northern blotting. In RNase protection assays, this cDNA probe proved unsuccessful for use as a riboprobe as it generated two protected fragments of smaller size (250 and 238 bases) instead of the expected protected fragment of 595 bases (Figure 4.9). This is most likely to be a result of a GC rich region in the DNA sequence that is susceptible to degradation by RNase due to weak hybridisation to its complementary sequence. In order to eliminate this problem, the cDNA sequence containing the first 3 exons of porcine IGFBP-3 was digested with suitable restriction enzymes and subcloned to generate three probes complementary to different regions of porcine IGFBP-3 mRNA. A riboprobe complementary to 208 bases in exon 1 of porcine IGFBP-3 (generated from p208BP3) did not protect any RNA species from porcine liver (Figure 4.11). The lack of RNA hybridisation is not clear. It is unlikely to be due to the DNA sequence, since the GC rich region previously eluded to is downstream from the sequence encoded by p208BP3. It may be that different hybridisation conditions are necessary for optimum hybridisation between the riboprobe generated from p208BP3 and porcine IGFBP-3 mRNA. Using p256BP3 to generate a riboprobe complementary to parts of exon 1 and 2 of porcine IGFBP-3 generated a protected fragment that was considerably smaller than the expected 256 bases (Figure 4.12). This was most likely a result of RNase digestion of weak RNA : RNA hybrids due to a GC rich sequence at the 3' end of exon 1. A exon 2 and 3 specific probe was generated as described in section 4.3.3. Transcription from the T3 promoter produced an antisense RNA probe 192 bases in length, which includes some sequence belonging to Bluescript® II SK+. In a RNase protection assay, the IGFBP-3 probe gives rise to one major band 139 bases in length, specific to porcine IGFBP-3 mRNA (Figure 4.13). Since this was the only one of the three subcloned fragments that was able to generate the results expected, this cDNA clone complementary to exons 2 and 3 of porcine IGFBP-3 was used in all subsequent assays to detect changes in IGFBP-3 mRNA expression.

The RNase protection assay was utilised in this study to detect changes in IGF-I and IGFBP-3 mRNA expression in different porcine tissues. It was necessary to ensure that the amount of RNA added to the hybridisation reaction was at an optimum. The intensity of the signal had to be within the linear concentration range but had to be strong enough in order to detect moderate decreases in mRNA expression but at the same time allow for the detection of increases in mRNA expression without saturating the amount of probe added to the reaction. Upon analysis of the concentration curves for both IGF-I *class 1* and IGFBP-3 mRNA expression (Figure 4.15 and 4.16), a quantity of RNA equating to 50 µg was chosen since this was near the mid range for both mRNA species. Similarly, for detection of 18S ribosomal RNA, a quantity of RNA equating to 10 µg was chosen. Amounts of RNA greater than this

rapidly saturated the available probe (Figure 4.17) introducing inaccuracies when quantitating gene expression. These quantities of RNA were used in all subsequent assays and provided consistent results throughout all experiments as measured by a control RNA sample analysed with every gel.

Optimisation of the RNase protection assay method for detection of IGF-I and IGFBP-3 mRNA and 18S RNA enables the regulation of IGF-I and IGFBP-3 mRNA expression to be examined. One of the aims of this study is to determine if IGF-I or LR^3IGF-I treatment in pigs alters the expression of IGF-I and/or IGFBP-3 mRNA due to reductions in plasma GH concentrations. In order to examine the effects of reduced GH levels on IGF-I and IGFBP-3 gene expression, its is firstly necessary to establish in which porcine tissues IGF-I and IGFBP-3 gene expression are regulated by GH. This is the focus of the next chapter.

CHAPTER 5

GROWTH HORMONE TREATMENT INCREASES LIVER IGF-I mRNA EXPRESSION AND LIVER AND KIDNEY IGFBP-3 mRNA EXPRESSION IN PIGS.

5.1 Introduction

The results presented in chapter 3 suggest that IGF-I and LR^{3}IGF-I treatment in pigs results in a decrease in average plasma GH levels and LR^{3}IGF-I treatment also reduces the amplitude of plasma GH pulses. This decrease in plasma GH levels is associated with a reduction in plasma IGF-I and IGFBP-3 levels. It was hypothesised that the decrease in plasma IGF-I and IGFBP-3 levels is a result of down regulation of mRNA expression brought about by the reduction in plasma GH. In order to study the expression of IGF-I and IGFBP-3 mRNA levels in different tissues in response to IGF-I or LR^{3}IGF-I treatment, it was necessary to obtain or produce suitable probes to measure mRNA expression utilising RNA protection by antisense probes. This work was described in the previous chapter. To prove the hypothesis that reduced levels of plasma GH result in a decrease in IGF-I and IGFBP-3 expression, it was first necessary to establish in which tissues IGF-I and IGFBP-3 expression are regulated by GH. This is the focus of the present chapter.

In growing pigs, GH treatment increases average daily weight gain (Weeden *et al.*, 1993), enhances protein accretion and reduces fat deposition (Campbell *et al.*, 1989). GH treatment has selective effects on some organs. It has been shown to increase pituitary (Andres *et al.*, 1993), kidney, heart, ovary and adrenal weights (Evock *et al.*, 1988; Bryan *et al.*, 1989; Bryan *et al.*, 1992). Many of the growth promoting actions of GH are mediated via IGF-I, and GH has been established as a major regulator of IGF-I gene expression. As a consequence, GH treatment increases plasma IGF-I levels in many different species. Hypophysectomized rats treated with GH show increased levels of plasma IGF-I and IGFBP-3 (Glasscock *et al.*, 1991). Mice made transgenic by the introduction of a ovine GH gene under the control of the metalothioneine promoter show elevated levels of plasma IGF-I and IGFBP-3 as well as increased expression of hepatic IGF-I and IGFBP-3 upon activation of the transgene (Chow *et al.*, 1994). GH also regulates the expression of IGF-I mRNA in rat skeletal muscle, heart (Isgaard *et al.*, 1989) and liver of adult (Glasscock *et al.*, 1991) and juvenile rats (Domenè *et al.*, 1993). In sheep, GH increases plasma IGF-I levels and liver IGF-I mRNA (Hua *et al.*, 1993). In the pig, plasma IGF-I and IGFBP-3 levels are elevated in GH treated animals (Coleman and Etherton, 1991; Owens *et al.*, 1990). At the level of gene expression, GH has been shown to regulate IGF-I in liver, (Grant *et al.*, 1991) and adipose tissue (Wolverton *et al.*, 1992) but not skeletal muscle (Grant *et al.*, 1991; Coleman *et al.*, 1994).

In the pig, one of two leader exons (exons 1 and 2) for IGF-I are alternatively spliced to exon 3 which encodes part of the mature IGF-I protein. Initiation of transcription at multiple dispersed start sites upstream of exon 1 and 2 yields two distinct IGF-I mRNAs, *class 1* and *class 2*, which differ in their precursor peptides. It has been shown that in the liver, expression of hepatic IGF-I *class 2* mRNA is more sensitive to changes that affect optimal growth than IGF-I *class 1* mRNA, and is closely correlated with circulating IGF-I levels and growth rates altered by energy manipulation (Weller *et al.*, 1993). Recent studies have shown that pGH treatment in pigs increases the expression of IGF-I *class 1* and *class 2* mRNA transcripts in liver and IGF-I *class 1* transcripts are also increased in semitendinosus muscle and adipose tissue, while expression of IGF-I *class 2* mRNA was only detected in liver (Brameld *et al.*, 1996).

In contrast, regulation of IGFBP-3 mRNA expression has not been studied extensively. In the rat, liver IGFBP-3 mRNA expression is also regulated by GH but appears to be less sensitive than IGF-I to circulating GH levels (Domenè *et al.*, 1993). To date there has been no study reporting the effects of GH administration on IGFBP-3 gene expression in porcine tissues.

The aim of this part of the study is to determine the effects of pGH treatment at a dose known to improve growth performance on the expression of IGF-I *class 1*, *class 2* and IGFBP-3 mRNA in liver, kidney, skeletal muscle (longissimus dorsi), stomach and small intestine of growing pigs.

5.2 Materials and Methods

5.2.1 Animals and animal maintenance

Pigs were obtained and housed at the Victorian Institute of Animal Science, Werribee, Victoria, Australia. The animals were individually penned in metabolism cages throughout the experiment and were meal fed ad libitum.

5.2.2 Experimental design

Ten female cross bred pigs (55 kg) were randomly allocated to one of two treatment groups. Animals received once daily injections of either saline or porcine growth hormone (70 µg/kg/day) (Bresatec Pty Ltd, Adelaide, Australia) for 4 days. On the last day of treatment, the daily dose was given as two injections 12 hours apart. One hour after the last injection, the animals were sacrificed and the pituitary was removed and weighed. Samples were taken from the left anterior lobe of the liver, left kidney including sections of the medulla and cortex, skeletal muscle from the P2 region (longissimus dorsi), small intestine (jejunum) and posterior stomach. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C until they were analysed. Animals were weighed daily during the course of the experiment.

The study was approved by the Victorian Institute of Animal Science Animal Experimentation Ethics Committee and the Animal Ethics Committee from the University of Adelaide.

5.2.3 Determination of average daily gain and feed intake

Pigs were weighed daily at 8 am on commercial livestock scales that had been incorporated into a metabolism cage. To determine feed intake, the amount of feed remaining after every 24 hour period was weighed and subtracted from the total daily feed supplied.

5.2.4 RNA isolation and RNase protection assay

RNA was extracted from tissues as described in section 4.2.18. High specific activity radioactive riboprobes for detection of IGF-I *class 1*, IGF-I *class 2* and IGFBP-3 mRNA were generated as described in section 4.2.20 and 4.2.21 and generation of a lower specific activity probe for detection of 18S ribosomal RNA was performed as described in section 4.2.22. DNA molecular markers were radioactively end labelled according to the method described in section 4.2.19. RNase protection assays were carried out as described in section 4.2.23 with the exception that 50 μg of total RNA was used for detection of IGF-I *class 1*, *class 2* and IGFBP-3 mRNA and 10 μg of total RNA was used for the detection of 18S ribosomal RNA.

5.2.5 Calculations and statistical analysis

All data was analysed using SigmaStat™ statistical software version 1.0 (Jandel Scientific Software, USA). Average daily gain (ADG) and feed : gain ratio were analysed using Student's t-test. The intensities of protected fragments corresponding to IGF-I *class 1*, *class 2* and IGFBP-3 mRNA were normalised and expressed as a percentage of the corresponding intensities for 18S ribosomal RNA protected fragments. To avoid inter-assay variation, all samples from the same tissue were analysed on one gel. Data were analysed for statistically significant differences using Student's t-test and are expressed as the mean ± SEM with $n = 5$.

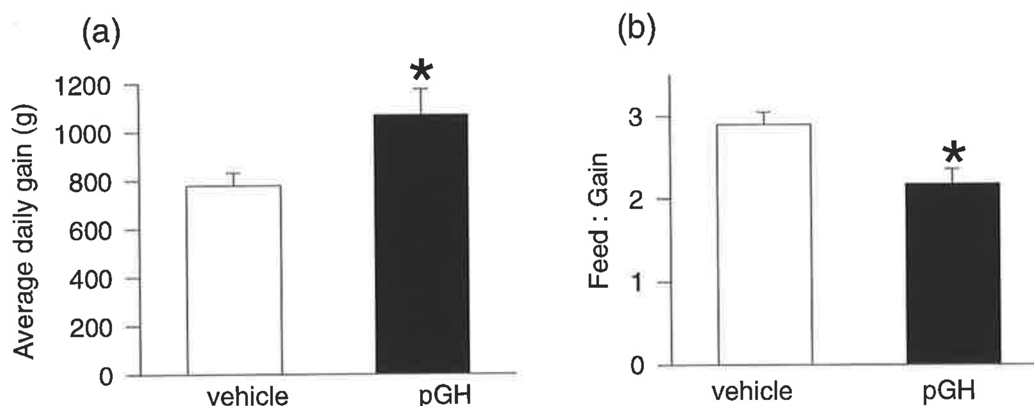
5.3 Results

5.3.1 Effects of pGH on growth rate and pituitary weight.

Administration of pGH for 4 days significantly increased average daily gain and decreased feed : gain ratio ($P < 0.05$) (Figure 5.1). Pituitary weights were not significantly different for animals receiving pGH (244.2 mg ± 6.4) relative to the control group (228.6 mg ± 12.9).

Figure 5.1:

Effect of vehicle or pGH treatment on average daily gain (a) and feed : gain ratio (b) over 4 days of treatment.



*Significant differences from the vehicle group are denoted by * P < 0.05.*

5.3.2 Effects of pGH on IGF-I class 1 and class 2 mRNA expression.

IGF-I *class 1* gene expression is represented by two protected fragments of 200 and 170 bases (Figure 5.2a). Autoradiographs from RNase protection assays to detect the steady state expression of IGF-I *class* 1 and IGF-I *class* 2 mRNA in porcine liver are shown in Figure 5.2 and the statistical interpretation of the results are presented in Figure 5.3. Treatment with pGH significantly increased the expression of IGF-I *class 1* mRNA in liver ($P < 0.02$) (Figure 5.3a). A third protected fragment of 147 bases representing a class of IGF-I transcript other than *class 1* was also increased by pGH treatment (Figure 5.2a) and could be detected in all tissues analysed.

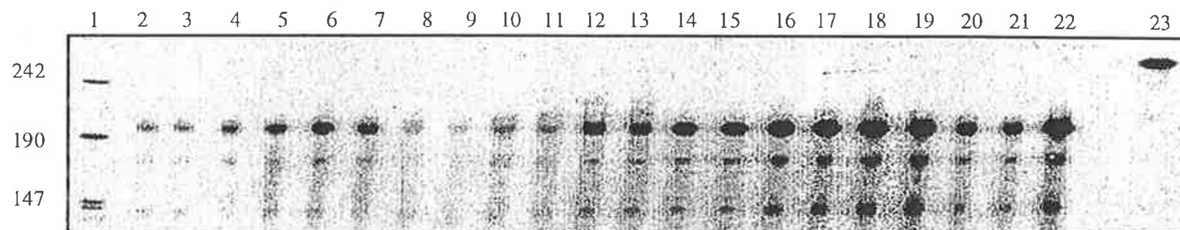
IGF-I *class 2* mRNA expression is predominantly found in the liver. It is represented by one protected fragment of 190 bases (Figure 5.2b). Treatment with pGH significantly increased the expression of IGF-I *class 2* mRNA ($P < 0.01$) (Figure 5.3b). An additional protected fragment of 147 bases representing a class of IGF-I transcript other than *class 2* was also increased by pGH treatment.

In kidney (Figure 5.4), skeletal muscle (Figure 5.5), stomach (Figure 5.6) and small intestine (Figure 5.7), IGF-I *class 1* mRNA expression was not altered by pGH treatment.

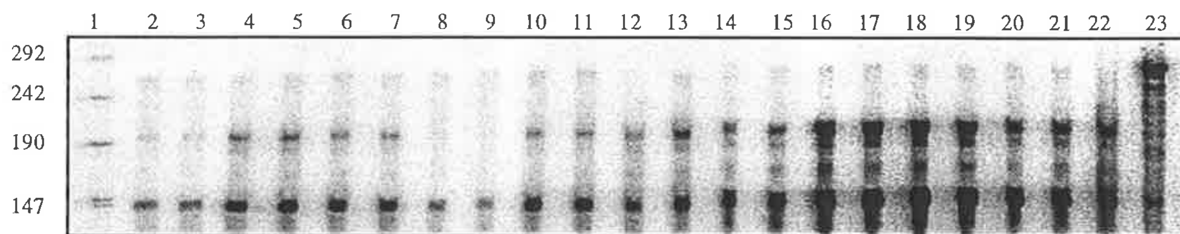
Figure 5.2:

Effect of pGH treatment on IGF-I *class 1* and IGF-I *class 2* mRNA expression in porcine liver

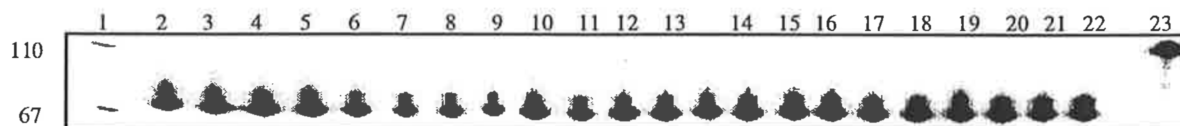
(a)



(b)



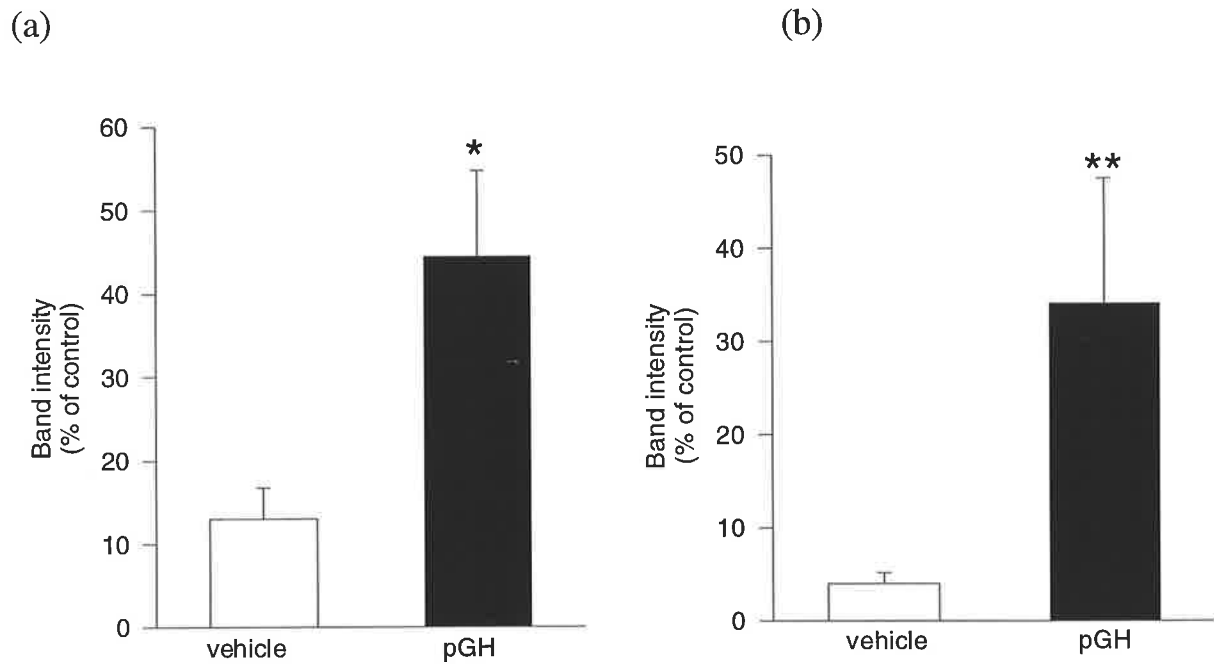
(c)



IGF-I class 1 (a), IGF-I class 2 (b) or 18S RNA (c) probes were hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs. Each sample was analysed in duplicate. Lane 22 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 23 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1.

Figure 5.3:

Graphical representation of IGF-I *class 1* (a) and IGF-I *class 2* (b) mRNA levels from livers of pGH treated pigs

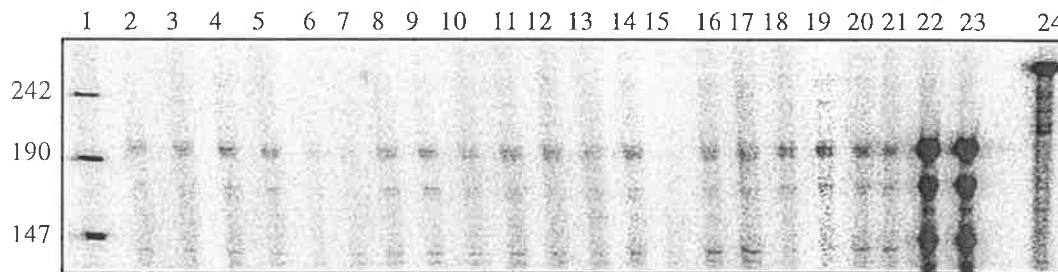


*Band intensities are quantitated using a phosphorimager and are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 5.2c). Significant differences from the vehicle group are denoted by * $P < 0.02$ and ** $P < 0.01$ and results are expressed as the mean ± SEM, n = 5 animals per treatment group.*

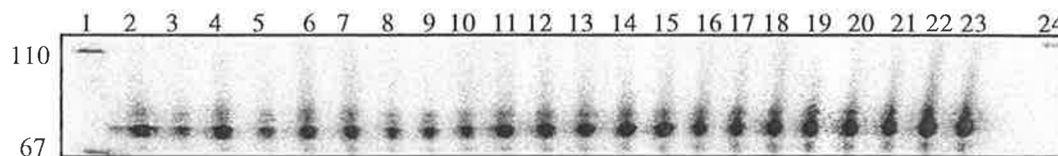
Figure 5.4:

Effect of pGH treatment on IGF-I *class 1* mRNA expression in porcine kidney

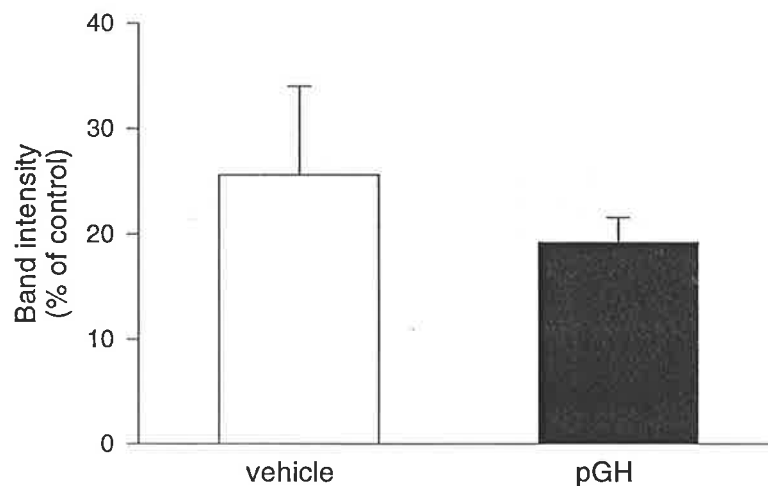
(a)



(b)



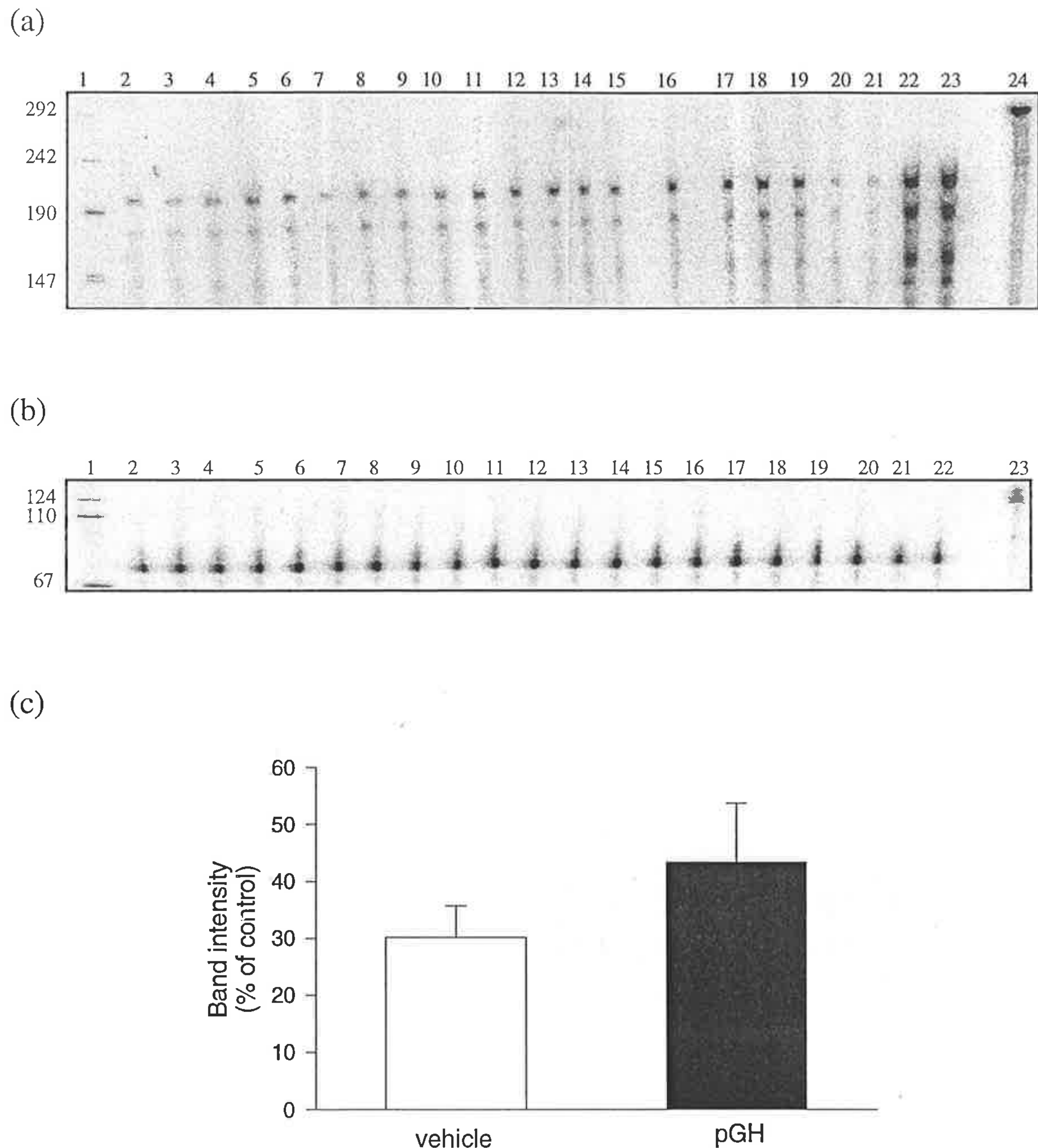
(c)



IGF-I class 1 (a) or 18S RNA (b) probes were hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs. Each sample was analysed in duplicate. Lanes 22 & 23 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM, n = 5 animals per treatment group (c).

Figure 5.5:

Effect of pGH treatment on IGF-I *class 1* mRNA expression in porcine muscle

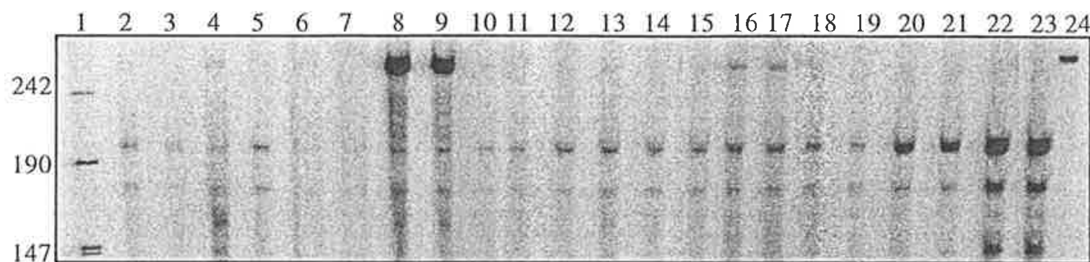


IGF-I class 1 (a) or 18S RNA probes were hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs. Each sample was analysed in duplicate. Lanes 22 & 23 (lane 22 only for b) represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 24 (a) and 23 (b) represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM, n = 5 animals per treatment group (c).

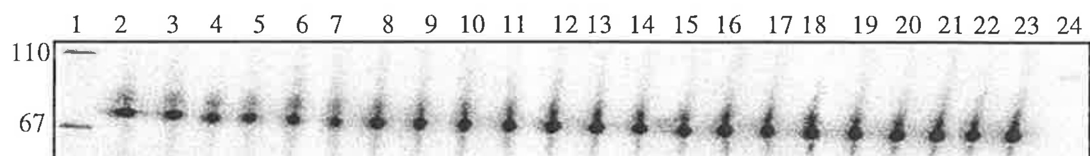
Figure 5.6:

Effect of pGH treatment on IGF-I *class 1* mRNA expression in porcine stomach

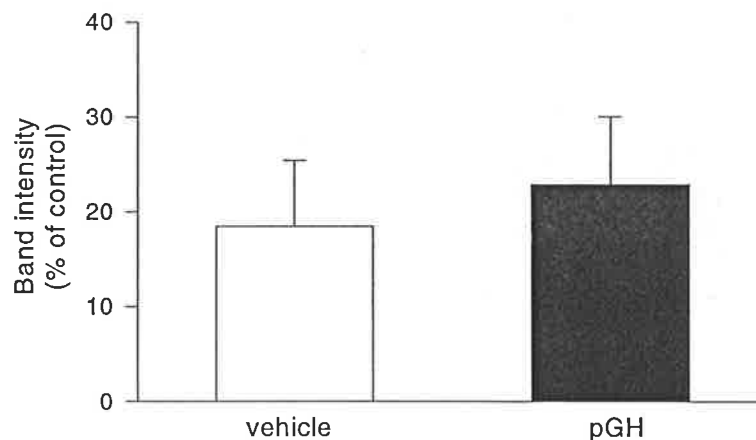
(a)



(b)



(c)

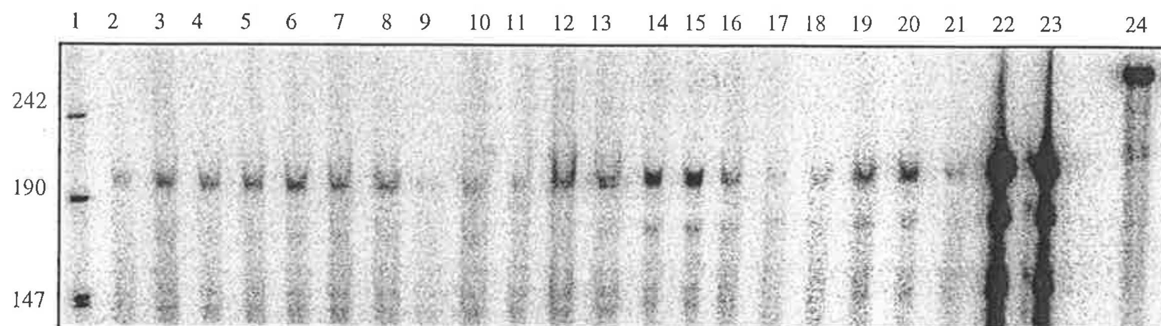


IGF-I class 1 (a) or 18S RNA (b) probes were hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs. Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM, n = 4 for vehicle and n = 5 for pGH treated pigs (c). The pig represented in lanes 8 & 9 was not included in the analysis due to poor RNase digestion.

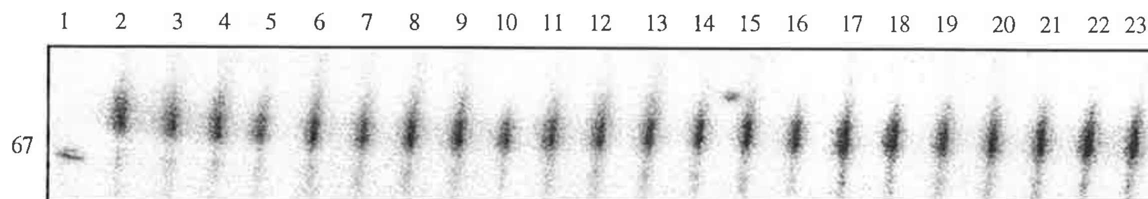
Figure 5.7:

Effect of pGH treatment on IGF-I *class 1* mRNA expression in porcine small intestine

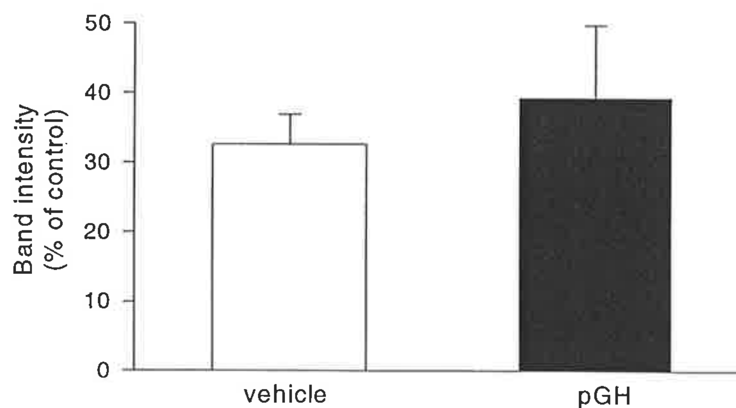
(a)



(b)



(c)



IGF-I class 1 (a) or 18S RNA (b) probes were hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs. Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM, n = 5 animals per treatment group (c).

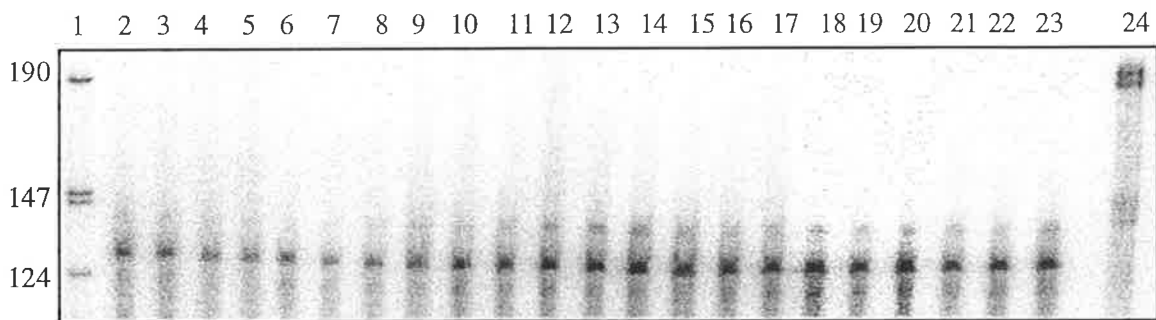
5.3.3 Effect of pGH treatment on IGFBP-3 mRNA expression.

IGFBP-3 gene expression is represented by one protected fragment of 139 bases. Autoradiographs from RNase protection assays to detect the expression of IGFBP-3 mRNA in different porcine tissues are shown in Figures 5.8 to 5.12. IGFBP-3 mRNA expression was significantly increased by pGH treatment in liver ($P < 0.03$) (Figure 5.8) and kidney ($P < 0.05$) (Figure 5.9) but not muscle (Figure 5.10), stomach (Figure 5.11) and small intestine (Figure 5.12).

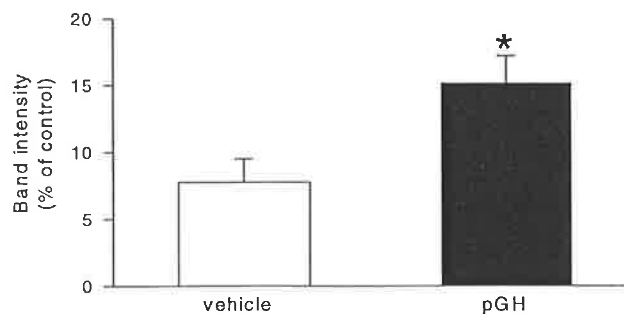
Figure 5.8:

Effect of pGH treatment on IGFBP-3 mRNA expression in porcine liver

(a)



(b)

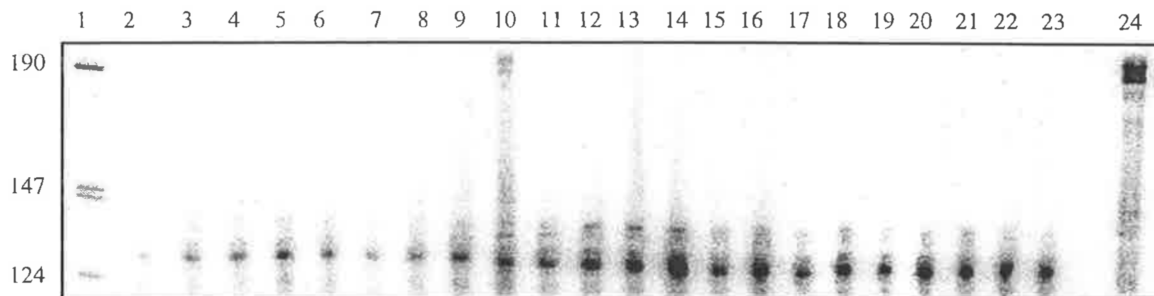


A porcine IGFBP-3 probe was hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs (a). Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 5.2c). Results are expressed as the mean ± SEM, n = 5 animals per treatment group (b).

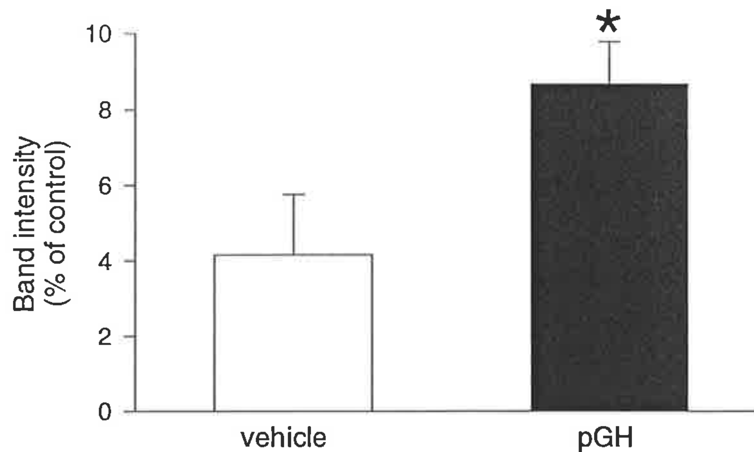
Figure 5.9:

Effect of pGH treatment on IGFBP-3 mRNA expression in porcine kidney

(a)



(b)

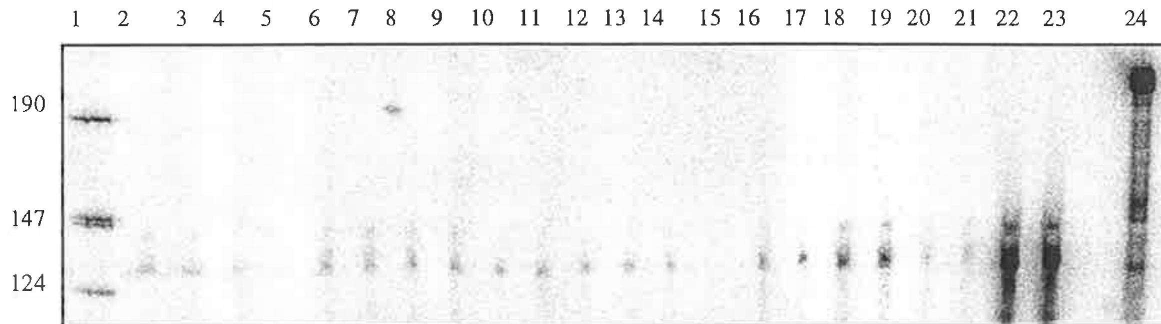


A porcine IGFBP-3 probe was hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs (a). Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 5.4b). Results are expressed as the mean ± SEM, n = 5 animals per treatment group (b).

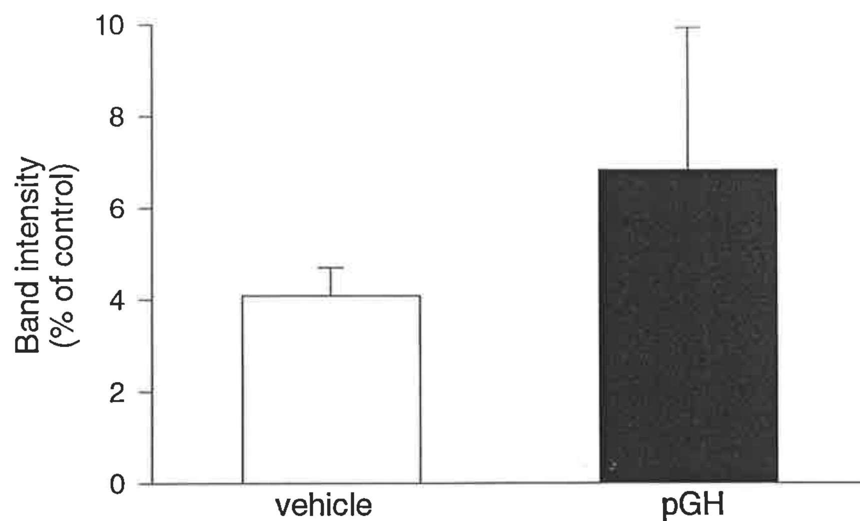
Figure 5.10:

Effect of pGH treatment on IGFBP-3 mRNA expression in porcine muscle

(a)



(b)

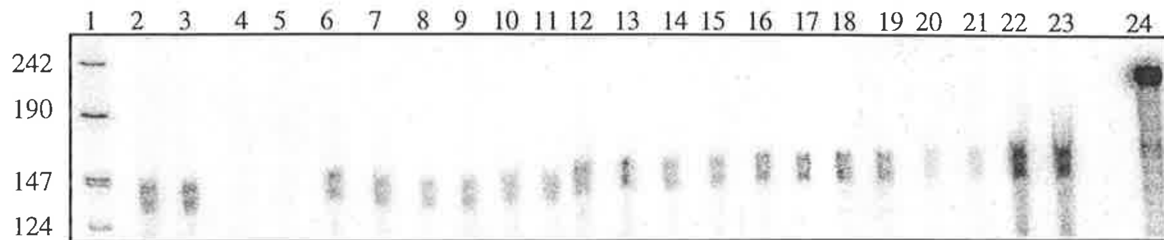


A porcine IGFBP-3 probe was hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs (a). Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 5.5b). Results are expressed as the mean ± SEM, n = 5 animals per treatment group (b).

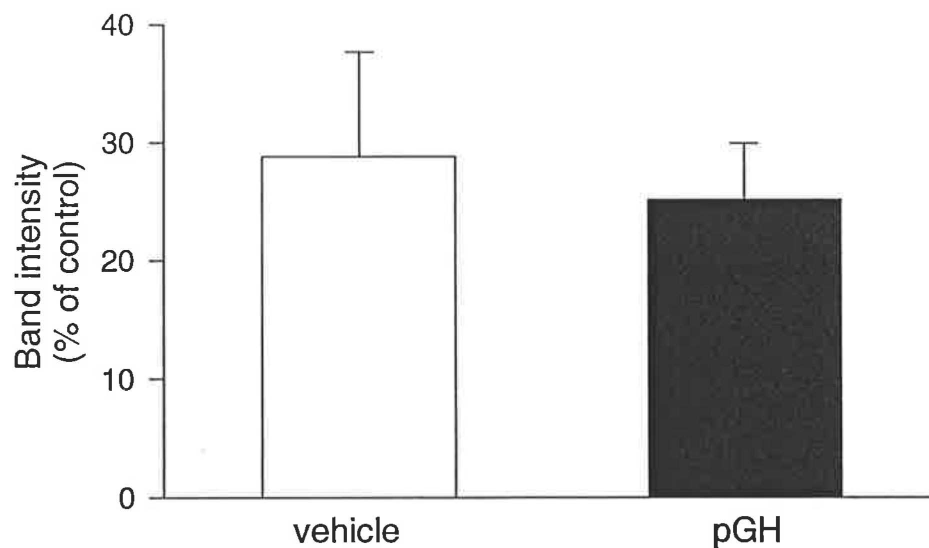
Figure 5.11:

Effect of pGH treatment on IGFBP-3 mRNA expression in porcine stomach

(a)



(b)

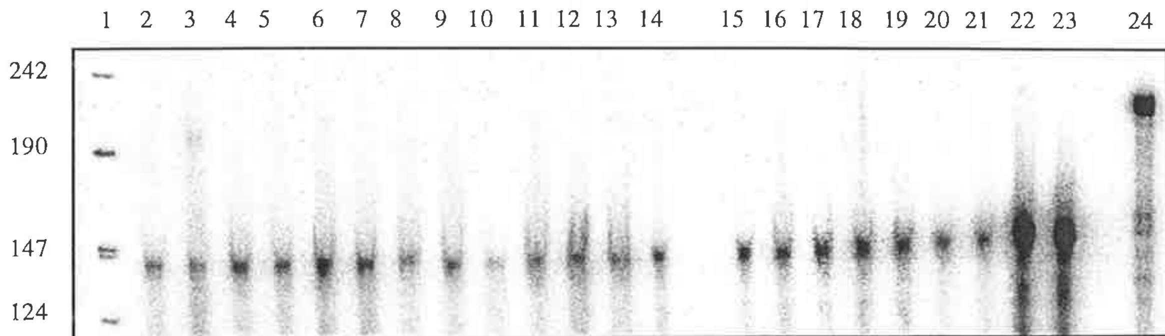


A porcine IGFBP-3 probe was hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs (a). Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 5.6b). Results are expressed as the mean ± SEM, n = 5 animals per treatment group (b).

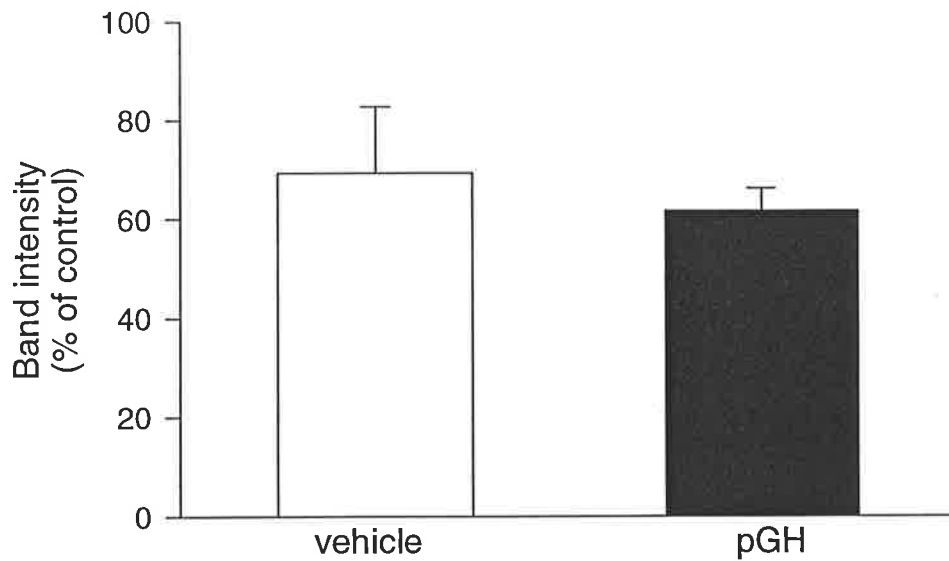
Figure 5.12:

Effect of pGH treatment on IGFBP-3 mRNA expression in porcine small intestine

(a)



(b)



A porcine IGFBP-3 probe was hybridised with total RNA from vehicle (lanes 2 - 11) or pGH (lanes 12 - 21) treated pigs (a). Each sample was analysed in duplicate. Lanes 22 & 23 represent control hybridisations to RNA extracted from the liver of pGH treated animals, and lane 24 represents a full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 5.7 b). Results are expressed as the mean ± SEM, n = 5 animals per treatment group (b).

5.3.4 Summary of results

A summary of the results obtained from the study described in this chapter is given in Table 5.1

Table 5.1:

Effect of pGH treatment on growth performance and IGF-I and IGFBP-3 gene expression

	pGH treatment
ADG	↑
Pituitary weight	↑
Liver IGF-I *class 1* mRNA	↑
Liver IGF-I *class 2* mRNA	↑
Liver IGFBP-3 mRNA	↑
Kidney IGF-I *class 1* mRNA	→
Kidney IGFBP-3 mRNA	↑
Muscle IGF-I *class 1* & IGFBP-3 mRNA	→
Stomach IGF-I *class 1* & IGFBP-3 mRNA	→
Small intestine IGF-I *class 1* & IGFBP-3 mRNA	→

Treatment groups are presented in the first row and parameters measured in the first column; → indicates no significant difference from vehicle treated pigs; ↑ indicates significant increase from vehicle treated pigs.

5.4 Discussion

The ability of pGH treatment to enhance growth performance in pigs has been well documented (Etherton *et al*, 1986; Campbell *et al*, 1988, Klindt *et al*, 1992). GH treatment increases average daily gain and feed : gain ratio (Weeden *et al.*, 1993) by enhancing protein accretion and reducing fat deposition in pigs (Campbell *et al.*, 1989). In the present study, pGH treatment at a dose of 70 μg/kg/day significantly increased ADG and improved feed efficiency as evident by a decrease in the feed : gain ratio of pGH treated pigs.

Long term treatment of pigs with pGH has been reported to increase anterior and posterior pituitary weights (Andres *et al.*, 1993; Campbell *et al.*, 1989) while decreasing GH concentrations in the anterior pituitary. In the present study, there were no differences in total pituitary weights between vehicle and pGH treated pigs. The conflicting results may be due to the short treatment period of the present study.

In porcine liver, IGF-I *class 1* and *class 2* gene expression are both increased by GH treatment. Pigs exhibiting an energy induced reduction in growth rate show decreased expression of liver IGF-I *class 1* and *class 2* mRNA (Weller *et al.*, 1994). These results, taken together with the present finding that increases in average daily gain are associated with increased expression of liver IGF-I *class 1* and *class 2* mRNA confirm that the actions of growth hormone on growth performance are mediated via IGF-I.

IGF-I *class 2* transcripts are more sensitive to changes in energy status than IGF-I *class 1* transcripts leading to a positive correlation between IGF-I *class 2* gene expression and plasma IGF-I levels (Weller *et al.*, 1994). In the present study, GH treatment resulted in a four fold increase in liver IGF-I *class 1* mRNA expression, while IGF-I *class 2* transcripts were increased eight fold. This supports previous findings that IGF-I *class 2* transcripts are the primary source of endocrine regulated IGF-I (Weller *et al.*, 1994). The virtual absence of IGF-I *class 2* transcripts in muscle (Weller *et al.*, 1994) and the absence of any detectable amount of IGF-I *class 2* transcripts in kidney suggests that the liver is the major source of endocrine regulated IGF-I. Recent studies have shown that pGH administration in pigs also increases IGF-I *class 1* mRNA expression in semitendinosus muscle and adipose tissue but not in longissimus muscle. In addition, IGF-I *class* 2 mRNA expression is also increased with pGH treatment in liver and is not detected in either muscle type nor adipose tissue (Brameld *et al.*, 1996).

IGF-I plays a role in the regulation of renal vascular resistance, renal blood flow and glomerular filtration rate. In states of acromegaly, the excess GH activity has been shown to

elevate renal blood flow and glomerular filtration rate (Parving *et al.*, 1978). Similarly, chronic administration of GH to normal subjects for one week raises glomerular filtration rate (Christiansen *et al.*, 1981), while acute intravenous infusion of GH has no effect on renal blood flow and glomerular filtration rate (Parving *et al.*, 1978). The effects of GH are thought to be exerted through IGF-I since acute intravenous infusion of IGF-I increases renal blood flow and glomerular filtration rate in rats (Hirschberg and Kopple, 1989) and humans (Hirschberg *et al.*, 1993). Both glomerular filtration rate and plasma IGF-I levels are decreased in states of GH deficiency, panhypopituitarism, feeding of low protein diets, starvation and malnutrition (Feld and Hirschberg, 1996). These findings indicate that in the kidney, IGF-I action is dependent on GH status. This is supported by the presence of GH receptors in the thick ascending limb of the loop of Henle and IGF-I receptors throughout the nephron (Feld and Hirschberg, 1996). In the present study, kidney IGF-I *class 1* transcription is not altered by GH treatment, and IGF-I *class 2* transcripts could not be detected using RNase protection assays. The present findings indicate that the effects of IGF-I on renal function in response to GH treatment must originate from systemically derived IGF-I. Kidney IGF-I mRNA levels are increased in states of renal hypertrophy induced by IGF-I administration (Guler *et al.*, 1988), GH treatment, surgical nephrectomy, streptozotocin induced diabetes and potassium depletion (El Nahas *et al.*, 1989; Flyvbjerg *et al.*, 1991). This rise in mRNA levels is only transient despite sustained hypertrophy and indicates that local IGF-I mRNA expression is not dependent on GH but may be increased to initiate nephron growth following hypertrophy.

GH treatment in healthy volunteers results in increased plasma IGF-I levels but decreased urinary excretion of IGF-I (Uemasu *et al.*, 1996). This could be a result of the increased half life of IGF-I due to its binding to plasma IGFBP-3, which is elevated with GH treatment. Alternatively, IGF-I may accumulate in the kidney due to increased tubular uptake of filtered IGF-I, and its degradation in the renal tubules (Flyvbjerg *et al.*, 1993). The accumulation of IGF-I in the kidney may be the reason for increased renal IGFBP-3 gene expression in the present study.

It seems that the growth promoting effects of GH on longissimus dorsi muscle are not due to increased local expression of IGF-I. In longissimus dorsi muscle, IGF-I expression is not regulated by GH or energy status (Coleman *et al.*, 1994; Grant *et al.*, 1991; Weller *et al.*, 1994). This suggests that in this muscle type, muscle growth is dependent on systemically derived IGF-I of hepatic origin. Indeed, GH receptor mRNA levels do not correlate with IGF-I mRNA levels and are inversely related to plasma IGF-I levels and growth rate in longissimus dorsi (Weller *et al.*, 1994). In contrast, in semitendinosus muscle, pGH treatment has been

shown to increase IGF-I *class 1* mRNA expression (Brameld *et al.*, 1996). The differences in response to pGH treatment in different muscles may be related to the different fibre types present. Longissimus dorsi muscle are a fast twitch muscle type (type IIB), while semitendinosus muscle are an intermediate muscle type (type IIC). It is interesting to note that pGH treatment increases GH receptor mRNA levels in both muscle longissimus dorsi and semitendinosus muscle, but IGF-I gene expression is only increased in the latter muscle type in response to pGH treatment. Thus the mechanism of regulating IGF-I gene expression by GH does not appear to be solely dependent on increases in GH receptor expression.

IGF-I mRNA has been found in small intestinal tissue of both suckling and adult rats (Dvorák *et al.*, 1996), and IGF-I and LR^3IGF-I infusion in rats has been shown to increase small intestinal weight and length as well as increasing crypt depth and villus height (Steeb *et al.*, 1994). Oral administration of IGF-I to newborn piglets has been shown to increase cell proliferation in small intestinal crypts (Xu *et al.*, 1994) and increase small intestinal weight and villus height without altering the circulating levels of IGF-I or IGF binding proteins (Burrin *et al.*, 1996). IGF-I receptors are found in the small intestine of pigs (Morgan *et al.*, 1996), rats (Young *et al.*, 1990) and rabbits (Termanini *et al.*, 1990), and in the pig, decrease with development. GH receptor gene expression has also been detected in stomach and small intestinal tissues of humans (Delehaye-Zervas *et al.*, 1994). These findings suggest an important role for IGF-I in the growth and development of the gastrointestinal tract. In the present study, IGF-I gene expression in the stomach and small intestine of pigs was low and did not respond to pGH treatment. These results indicate that regulation of IGF-I in these organs is of a auto/paracrine nature, and that GH effects are not mediated through IGF-I in theses organs.

GH treatment increases plasma IGFBP-3 and IGF-I levels in rats (Clemmons *et al.*, 1989), humans (Laron, 1993) and pigs (Walton and Etherton, 1989). In transgenic mice that express IGF-I in the absence of GH, plasma IGFBP-3 levels are increased, suggesting that IGF-I can regulate the expression of IGFBP-3 in the absence of GH (Camacho-Hubner *et al.*, 1991). Regulation of IGFBP-3 mRNA expression by GH has not been previously examined in the pig. IGFBP-3 mRNA has been detected in porcine gonadal tissue, brain, liver, muscle, lung and kidney (Shimasaki *et al.*, 1990; Lee *et al.*, 1993b). In the rat, the kidney and liver are the major source of IGFBP-3 (Albiston and Herington, 1992). This correlates with the present study in the pig, where IGFBP-3 gene expression was most abundant in liver and kidney and increased with GH treatment. Whether this is a direct effect of GH or a result of increased expression of IGF-I is not clear. IGF-I type 1 and GH receptors are present in both liver and kidney (Lee *et al.*, 1993b) hence either hormone may stimulate IGFBP-3 expression. In

hypophysectomized rats, liver IGF-I and IGFBP-3 mRNA expression are reduced while GH treatment restored both IGF-I and IGFBP-3 mRNA levels (Albiston and Herington, 1992). The effect of GH treatment on IGFBP-3 expression can not be attributed to GH alone since IGF-I levels are also depleted by hypophysectomy and then restored by GH treatment.

It is clear that GH plays an important role in IGF-I and IGFBP-3 gene expression. In the pig, the major source of endocrine regulated IGF-I appears to be the liver, although IGF-I expression has also been shown to be regulated by GH in adipose tissue (Wolverton *et al.*, 1992) and semitendinosus muscle (Brameld *et al.*, 1996). In the liver, IGF-I *class 2* transcripts are more sensitive to changes in growth rate and GH levels (Weller *et al.*, 1994; Brameld *et al.*, 1996), although a subpopulation of transcripts termed IGF-I *non class 1* and *non class 2* also appear to be regulated by GH (Figure 5.1). IGFBP-3 gene expression is also sensitive to GH status in liver and kidney. Whether this is a direct effect of GH or indirectly through IGF-I is not clear. In the pig, IGF-I and IGFBP-3 mRNA expression are not affected by GH treatment in muscle, stomach or small intestine.

In chapter 3 I have shown that treatment with LR^3IGF-I reduces average plasma GH levels and the amplitude of the GH pulses, as well as plasma IGF-I and IGFBP-3 levels. IGF-I treatment only suppresses average plasma GH levels. It was hypothesised that the decrease in average plasma GH levels and amplitude of the GH pulses was responsible for the reduction in plasma IGF-I and IGFBP-3 levels by reduced GH-induced mRNA synthesis. In order to test this hypothesis it was necessary to establish in which tissues IGF-I and IGFBP-3 were regulated by GH. This was the focus of the present chapter. In this part of the study, I have shown that IGF-I mRNA levels are regulated by GH in liver and IGFBP-3 mRNA levels are regulated by GH in liver and kidney. Consequently, in the pig, the liver and kidney are appropriate organs to study the expression of IGF-I *class 1* and *class* 2 and IGFBP-3 mRNA expression in response to IGF-I or LR^3IGF-I treatment. This will be the subject of the next chapter.

CHAPTER 6

IGF-I AND LR^{3}IGF-I TREATMENT DECREASES LIVER IGF-I BUT NOT IGFBP-3 mRNA EXPRESSION IN PIGS.

6.1 Introduction

Pigs treated with LR^{3}IGF-I exhibit decreased average daily weight gain and feed intake. Results presented in chapter 3 of this thesis show that this reduction in growth performance is associated with a reduction in plasma levels of GH, IGF-I, IGFBP-3 and insulin, whereas glucose levels remain unchanged. Treatment with IGF-I has similar effects in that plasma GH and insulin levels are also reduced in these animals. There are several possible explanations for these observations. The exogenously administered IGF peptides may be having metabolically toxic effects on pigs. The transport of glucose into cells is facilitated by IGF-I which may result in hypoglycaemia. This increased uptake of glucose can produce an efflux of potassium from the extracellular fluid resulting in hypokalemia. Metabolic acidosis may also be a cause of metabolic toxicity and is a result of the formation of excessive amounts of metabolic acids due to increased protein breakdown or decreased protein synthesis. However, IGF-I or LR^{3}IGF-I treatment does not alter nitrogen retention in pigs, indicating that protein breakdown is not affected by IGF treatment (Walton *et al.*, 1993). Plasma urea levels are an indicator of amino acid degradation, which occurs when there is an excess of amino acids not needed for protein synthesis. As amino acids are broken down, ammonium ions are formed, which are utilised in the biosynthesis of nitrogen compounds. The excess ammonium ions are converted into urea which is then excreted in the urine. In metabolic toxicity, a reduction in protein synthesis and thus an increase in plasma urea levels is expected. This has not been observed in pigs treated with IGF-I or LR^{3}IGF-I, where plasma urea levels were not different to those of untreated animals (Walton *et al.*, 1993). Creatinine is another indicator of amino acid breakdown and is the excretory product of phosphocreatine, a storage form of high energy phosphate in skeletal muscle, which is converted to ATP and creatinine when additional energy is required. Plasma creatinine levels are also not affected by IGF treatment (Walton *et al.*, 1993).

Alternatively, exogenous administration of IGF peptides may be producing a negative effect on IGF-I and/or IGFBP-3 gene expression by inhibiting plasma GH levels. In the pig, treatment with pGH has been shown to have a stimulatory effect on IGF-I mRNA expression in liver, kidney, adipose tissue and semitendinosus muscle (Grant *et al.*, 1991; Wolverton *et al.*, 1992; Coleman *et al.*, 1994; Brameld *et al.*, 1996; see chapter 5), while pGH treatment increases IGFBP-3 mRNA expression in liver and kidney. Treatment with pGH has no effect on IGF-I or IGFBP-3 mRNA expression in longissimus dorsi muscle, stomach or small

intestine (chapter 5). Thus expression of IGF-I and IGFBP-3 mRNA is dependent on plasma GH levels in several different porcine tissues.

IGF-I and LR^3IGF-I treatment reduce plasma GH levels and LR^3IGF-I treatment also reduces the magnitude of the pulsatile GH spikes. This is associated with a reduction in plasma IGF-I and IGFBP-3 levels. Whether the reduction in plasma GH levels has an effect on IGF-I and IGFBP-3 mRNA levels in tissues where these mRNAs are regulated by GH is not known. IGF-I may also be regulating its own expression through a negative feedback mechanism. These effects are difficult to examine in normal animals where the endocrine effects on IGF-I expression can not be separated from the auto/paracrine effects and can therefore not be examined in the present experiment.

The aim of the present study is to determine the effects of IGF-I or LR^3IGF-I treatment on IGF-I *class 1*, IGF-I *class 2* and IGFBP-3 mRNA expression in porcine liver and kidney. Expression of IGF-I and IGFBP-3 mRNAs in longissimus dorsi, stomach and small intestine are not examined since the expression of these mRNAs have been shown to be independent of GH levels (chapter 5).

6.2 Materials and Methods

6.2.1 Peptides and plasmids

Recombinant human IGF-I and LR^3IGF-I were supplied by GroPep Pty. Ltd., Adelaide, SA, Australia. Plasmids pRP1 and pRP2 were a gift from Dr Stewart Gilmour (Babraham Institute, Cambridge, UK). Plasmid p131BP3 was generated as described in chapter 4 section 4.2.17. pT718SRNA was purchased from Ambion Inc., Austin, Texas, USA).

6.2.2 Enzymes

Sal I and Bam HI were obtained from Progen Industries Ltd., Darra, Qld, Australia. Proteinase K, RQ1 RNase-free DNase and rRNasin were obtained from Promega Corporation, Madison, WI, USA. RNase A and RNase T1 were obtained from Sigma Chemical Company, St. Louis, MO, USA.

6.2.3 Radionucleotides

α-^{35}S-dATP, α-^{32}P-UTP and γ-^{32}P-dATP were obtained from Bresatec Ltd, Adelaide, Australia. Na-I^{125} was obtained from Amersham Australia Pty Ltd., NSW, Australia.

6.2.4 Chemicals

All chemicals were of analytical reagent grade and were obtained from Sigma Chemical Company, St. Louis, MO, USA, USA, Merk Pty Ltd, Kilsyth, Victoria, Australia or Ajax Chemicals, Auburn, NSW, Australia.

6.2.5 Animals and animal maintenance

Pigs (55 kg) were obtained and housed at the Victorian Institute of Animal Science, Werribee, Victoria, Australia. The animals were individually penned in metabolism cages throughout the experiment and were fed 110g/kg body weight per day.

6.2.6 Experimental design

Twelve female cross bred pigs (55 kg) were surgically prepared under halothane (May & Baker, West Footscray, Victoria, Australia) anaesthesia with 2 indwelling catheters and placed into metabolism cages 7 days before the commencement of treatment. During surgery, all pigs received an intra muscular injection of a long acting antibiotic, oxytetracycline (May & Baker, West Footscray, Victoria, Australia) and received medicated feed (1 g/kg terramycin, May & Baker, West Footscray, Victoria, Australia) throughout the study. Animals were randomly allocated to one of three treatment groups and received via intravenous infusion either saline (150 mM NaCl), IGF-I or LR^3IGF-I for 4 days. Travenol ® infusor pumps (Travenol Laboratories Inc., Deerfield, IL, USA) were changed every 24 hours and contained 10.4 mg of IGF peptides diluted in 50 ml saline. The delivery rate was 2 ml/hr, which equated to a dose of 180 µg/kg/day. Blood samples were taken at -24, -16, -8, 0, 8, 16, 24, 32, 48, 72 and 96 h. At the completion of the treatment period, animals were sacrificed and tissue samples were taken from the left anterior lobe of the liver and left kidney, snap frozen in liquid nitrogen and stored at -80°C until analysed.

The study was approved by the Victorian Institute of Animal Science Animal Experimentation Ethics Committee and the Animal Ethics Committee from the University of Adelaide.

6.2.7 Determination of plasma IGF-I concentration

Iodination of human IGF-I for use in IGF-I radioimmunoassay was performed as described in chapter 3 section 3.2.6. IGF peptides were separated from the IGF binding proteins by acid gel-filtration as described in chapter 3 section 3.2.7.1 and plasma IGF-I concentrations were determined by radioimmunoassay as described in chapter 3 section 3.2.7.2.

6.2.8 Western ligand blot analysis of porcine plasma

A 10 µl aliquot of plasma was mixed with 120 µl of 4 x loading buffer (3.02 % (w/v) Tris base, 8 % (w/v) sodium dodecyl sulphate, 20 % (v/v) glycerol, 0.004% (w/v) bromophenol blue, pH 6.8) and incubated at 65°C for 15 minutes. Molecular weight ^{14}C-labelled "rainbow" markers (Amersham Australia Pty Ltd., NSW, Australia) and 10 µl foetal calf serum (Gibco Laboratories, Chagrin Falls, OH, USA) were treated in the same manner. IGF binding proteins were separated by loading 25 µl of the heated plasma sample onto SDS-PAGE gels and electrophoresed using an LKB 2050 Midget electrophoresis unit (LKB Produkter AB, Bromma, Sweden). The gels contained stacking and separating gel components. Separating gels were prepared by mixing 12.5 % (w/v) acrylamide, 0.31 % (w/v) methyl bis-acrylamide, 0.1 % (w/v) sodium dodecyl sulfate and 0.38 M Tris-HCl, pH 6.8 and was set by the addition of 0.04 % (w/v) ammonium persulfate and 0.05 % (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED). The stacking gel was poured on top of the polymerised separating gel and consisted of 4 % (w/v) acrylamide, 0.1 % (w/v) methyl bis-acrylamide, 0.1 % (w/v) sodium dodecyl sulphate and 0.13 M Tris-HCl, pH 8.8 and was set by the addition of 0.01 % (w/v) ammonium persulfate and 0.05 % (v/v) TEMED. Electrophoresis was carried out at 15 mA per gel for approximately 2 hours.

Following electrophoresis, proteins were transferred from gels to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a Multiphor II electrophoresis system (Pharmacia LKB Biotechnology, Uppsala, Sweden). A discontinous buffer system was constructed as follows: six pieces of 3MM chromatography paper (Whatmann Inc, Clifton, NJ, USA) soaked in anode solution 1 (0.3 M Tris, pH 10.4) were placed on the anode electrode gel. On top of this were placed 3 pieces of chromatography paper soaked in anode solution 2 (25 mM Tris, pH 10.4) followed by nitrocellulose prewetted in anode solution 2. The gel was placed in contact with the nitrocellulose and covered with 9 pieces of chromatography paper soaked in cathode solution (4 mM 6-amino-n-hexanoic acid, pH 7.6). The cathode electrode plate was placed over the stack and transfer was carried out for 1 hour at 0.8 mA/cm^2 of gel surface area.

At the completion of transfer, the nitrocellulose membrane was washed in a solution of Triton X-100, 1 % (v/v), 0.15 M NaCl, 10 mM Tris base, pH 7.4 for 30 minutes at room temperature with gentle agitation. To block non-specific binding sites, the nitrocellulose was soaked for at least 2 hours in saline containing 0.1 % (v/v) Tween 20 and 1 % (w/v) bovine serum albumin. The nitrocellulose was transferred to a fresh batch of the same solution

containing approximately 500,000 cpm of ^{125}I-IGF-I and hybridised overnight at room temperature with gentle agitation. Excess radioligand was removed by 4 x 20 minute washes in 0.15 mM NaCl, 0.1 % (v/v) Tween 20. The nitrocellulose was air dried, wrapped in plastic film and exposed to a phosphorimager screen.

6.2.9 Analysis of IGF-I class 1, IGF-I class 2 and IGFBP-3 gene expression

Total RNA was extracted from liver and kidney as described in chapter 4 section 4.2.18 and the integrity of the RNA was checked by running aliquots on 1 % agarose gels as described in section 4.2.10. Radioactive molecular weight markers were generated as described in chapter 4 section 4.2.19. Riboprobes were generated as described in chapter 4 section 4.2.20 for the detection of IGF-I *class 1* and IGF-I *class 2* mRNA, section 4.2.21 for the detection of IGFBP-3 mRNA and section 4.2.22 for the detection of 18 S ribosomal RNA expression. RNase protection assays were carried out as described in section 4.2.23 with the exception that 50 μg of total RNA was used for detection of IGF-I *class 1*, *class 2* and IGFBP-3 mRNA and 10 μg of total RNA was used for the detection of 18S ribosomal RNA.

6.2.10 Statistical analysis

All data was analysed using SigmaStat™ statistical software version 1.0 (Jandel Scientific Software, USA). Plasma IGF-I concentrations were analysed by one way analysis of variance and significant differences were determined by all pairwise comparison using Bonferroni's t-test and are expressed as the mean ± SEM with n = 4. For detection of changes in IGF binding protein profiles, relevant band intensities (measured as integrated volume in arbitrary phosphorimager-derived units) were analysed for statistically significant differences using Student-Newman-Keul's t-test and are expressed as the mean ± SEM with n = 4. For analysis of mRNA expression, the intensities of protected fragments corresponding to IGF-I *class 1*, *class 2* and IGFBP-3 mRNA were normalised to expression of 18S RNA and expressed as a percentage of the corresponding intensities for 18S ribosomal RNA protected fragments. To avoid interassay variations, all samples from the same tissue were analysed on one gel. Data were analysed by one way analysis of variance and significant differences were determined by all pairwise comparison using Bonferroni's t-test and are expressed as the mean ± SEM with n = 4.

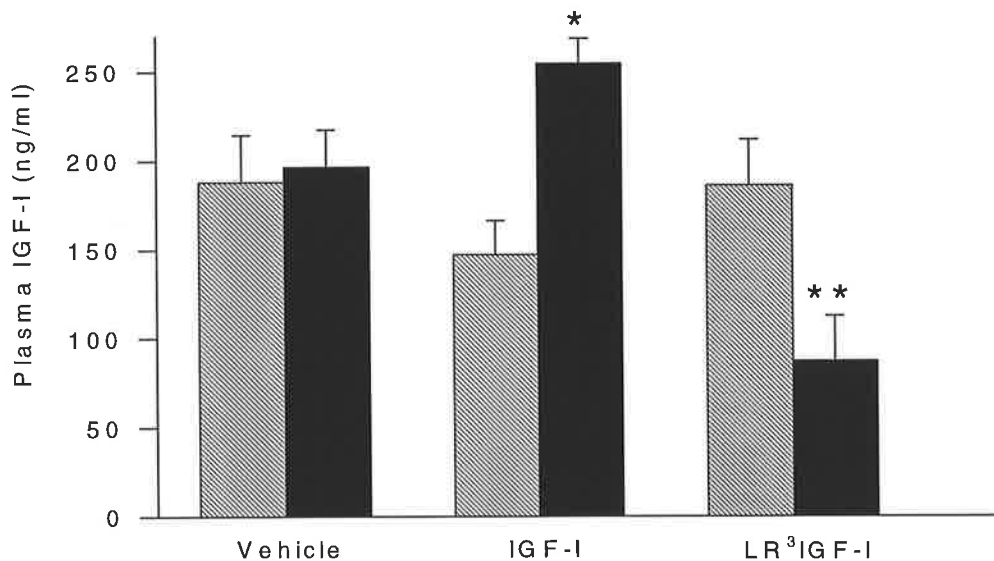
6.3 Results

6.3.1 Plasma IGF-I concentrations

Plasma IGF-I concentrations were measured in blood samples taken at the start and after 96 h of treatment (Figure 6.1). As expected, groups receiving IGF-I showed an increase in plasma IGF-I levels. In contrast, endogenous plasma IGF-I levels were significantly decreased by LR^3IGF-I treatment. These results are in agreement with the results described in chapter 3 (see Figure 3.1).

Figure 6.1:

Plasma IGF-I concentration measured at the start (hatched bars) and after 96 hours of treatment (solid bars)



*Results are expressed as the mean ± SEM for 4 animals in each treatment group. Significant differences are denoted by * indicating a significant increase in plasma IGF-I compared to the vehicle treated group ($P < 0.005$) and ** indicating a significant decrease in plasma IGF-I compared to the vehicle treated group ($P < 0.05$).*

6.3.2 Plasma IGF binding protein profiles

Plasma IGF binding proteins were analysed by western ligand blot (Figure 6.2). Three bands were observed with molecular weights of 43, 39, and 34 kDa. The 43 and 39 kDa bands have previously been identified by immunoprecipitation as a glycosylated doublet of porcine IGFBP-3 (Walton and Etherton, 1989). The intensities of the 43 and 39 kDa bands were measured using a phosphorimager, and the intensities compared between samples taken at the start and after 96 hours of treatment. Treatment with IGF-I (Figure 6.2a) had no significant effect on plasma IGFBP-3 levels (Figure 6.2b), but treatment with LR^3IGF-I (Figure 6.2 c) significantly decreased plasma IGFBP-3 levels (Figure 6.2d). The decrease in plasma concentrations of IGFBP-3 is consistent with results from a previous study discussed in chapter 3, where LR^3IGF-I treatment significantly decreased plasma levels of IGFBP-3 as measured by RIA (see Figure 3.2).

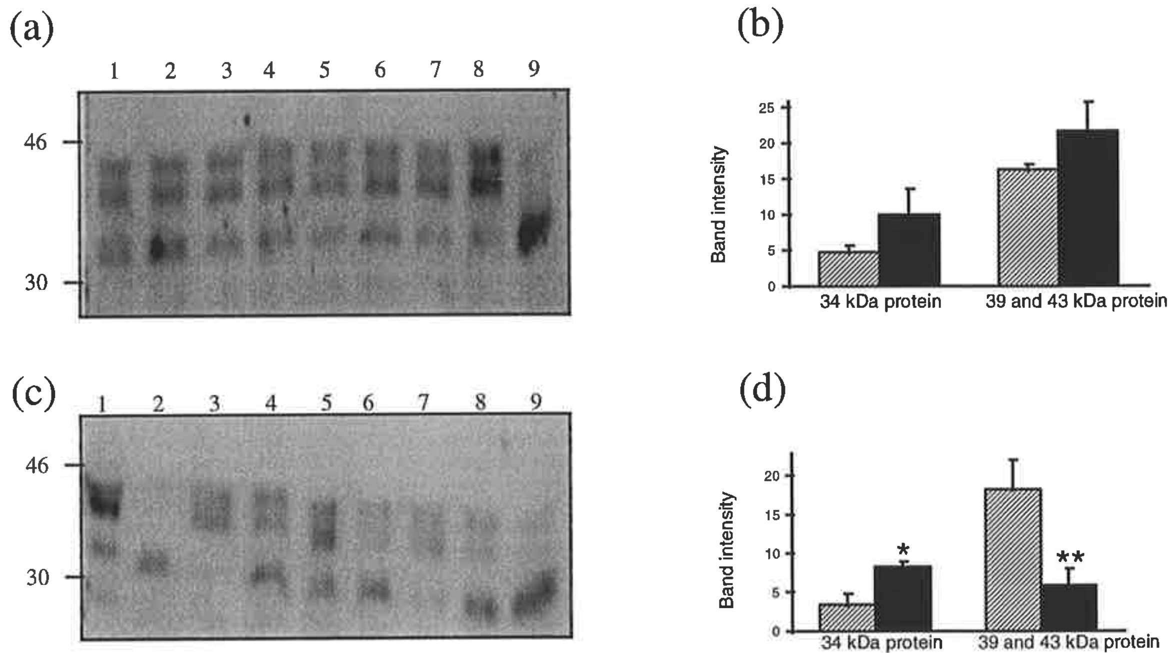
The porcine 34 kDa band has been shown to be immunologically related to rat IGFBP-2 (Coleman and Etherton, 1991; Lee *et al.*, 1991) and was significantly increased by LR^3IGF-I treatment (Figure 6.2c and d).

6.3.3 Effects of IGF-I and LR^3IGF-I on IGF-I class 1 and class 2 mRNA expression.

IGF-I *class 1* gene expression is represented by two protected fragments of 200 and 170 bases. Treatment with IGF-I or LR^3IGF-I had no significant effect on the expression of IGF-I *class 1* mRNA in liver, although a trend toward decreased levels of IGF-I *class 1* mRNA for both treatment groups was evident when compared to untreated animals (Figure 6.3).

Figure 6.2:

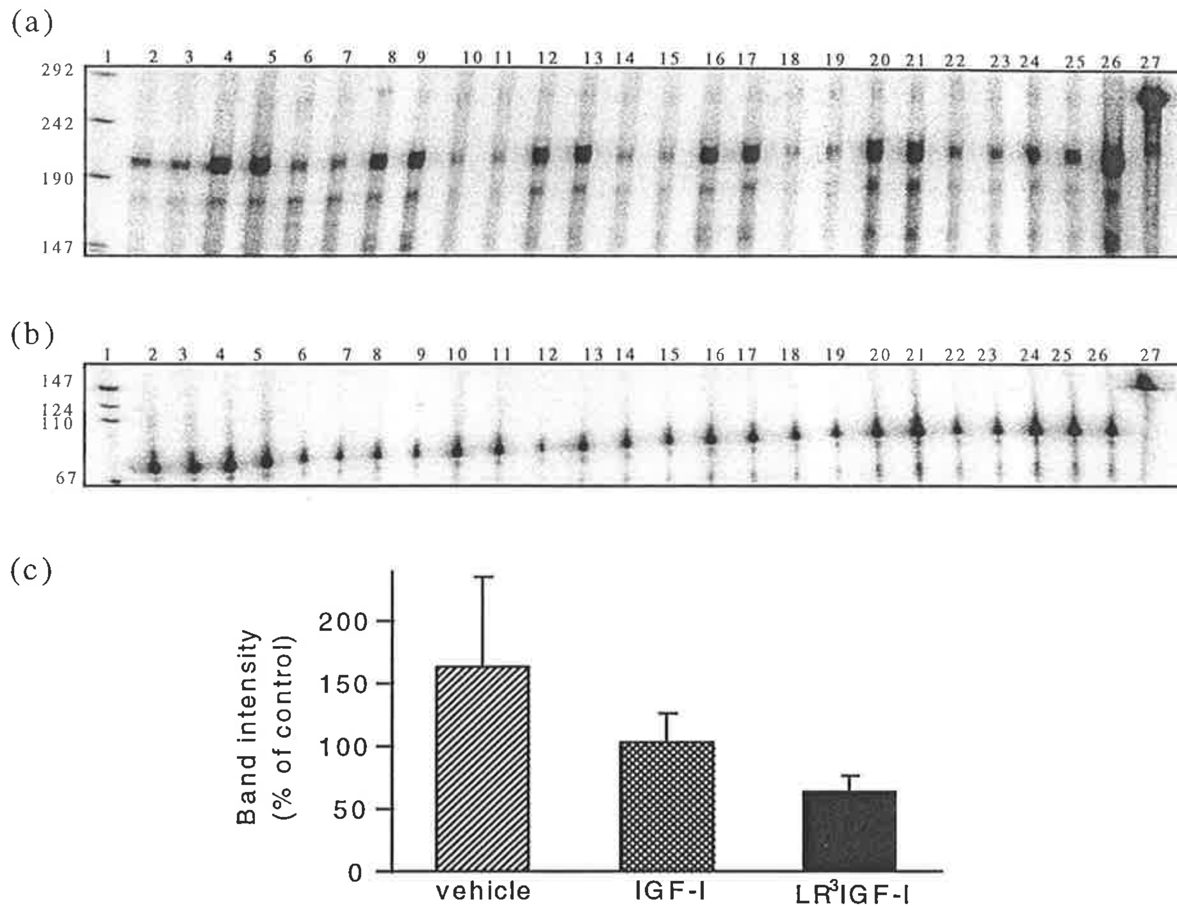
IGF binding protein profiles from pigs treated with IGF-I or LR³IGF-I



*Representative autoradiograms of western ligand blots containing 2 μl of plasma from individual pigs before (lanes 1, 3, 5, and 7) and after (lanes 2, 4, 6 and 8) 96 hours of treatment with either IGF-I (a) or LR³IGF-I (c). Lane 9 represents a 2 μl foetal calf serum as a control and molecular weight markers are indicated in kDa. Results are expressed as the mean ± SEM for 4 animals in each treatment group. Significant differences are denoted by * indicating a significant increase in plasma levels of the 34 kDa protein band (IGFBP-2) compared to pretreatment values ($P < 0.05$) and ** indicating a significant decrease in plasma levels of the 39 and 43 kDa protein bands (IGFBP-3) compared to pretreatment values ($P < 0.05$).*

Figure 6.3:

Effects of IGF-I and LR³IGF-I treatment on IGF-I *class 1* mRNA expression in porcine liver

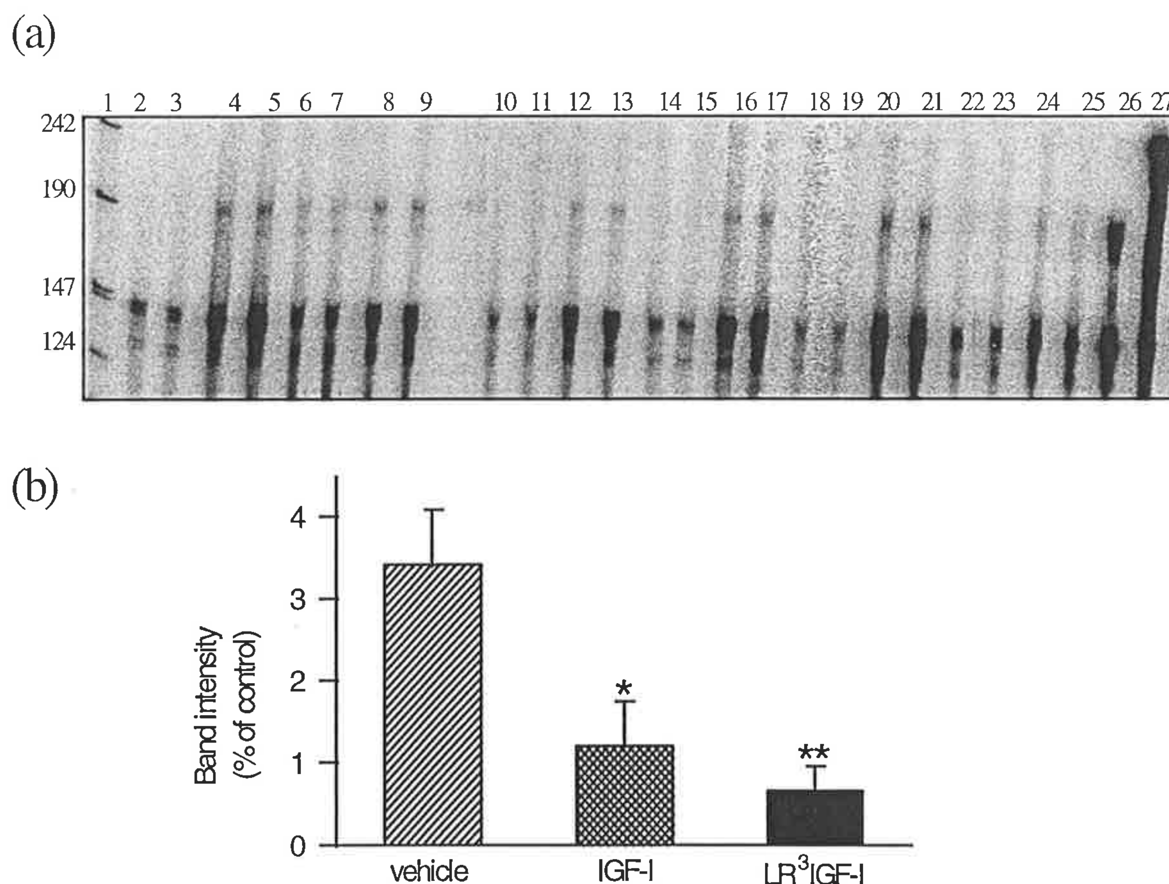


IGF-I class 1 probe (a) or 18S RNA probe (b) were hybridised with total liver RNA from vehicle (lanes 2 - 9), IGF-I (lanes 10 - 17) or LR³IGF-I treated pigs (lanes 18 - 25). Each sample was analysed in duplicate. Lane 26 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 27 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM for 4 animals per treatment group (c).

IGF-I *class 2* mRNA expression is represented by one protected fragment of 190 bases. A autoradiogram from RNase protection assays to detect the expression of IGF-I *class 2* mRNA is shown in Figure 6.4. Both IGF-I and LR^{3}IGF-I treatment significantly decreased the expression of IGF-I *class 2* mRNA in liver (P < 0.05 and P < 0.01 respectively).

Figure 6.4:

Effects of IGF-I and LR^{3}IGF-I treatment on IGF-I *class 2* mRNA expression in porcine liver

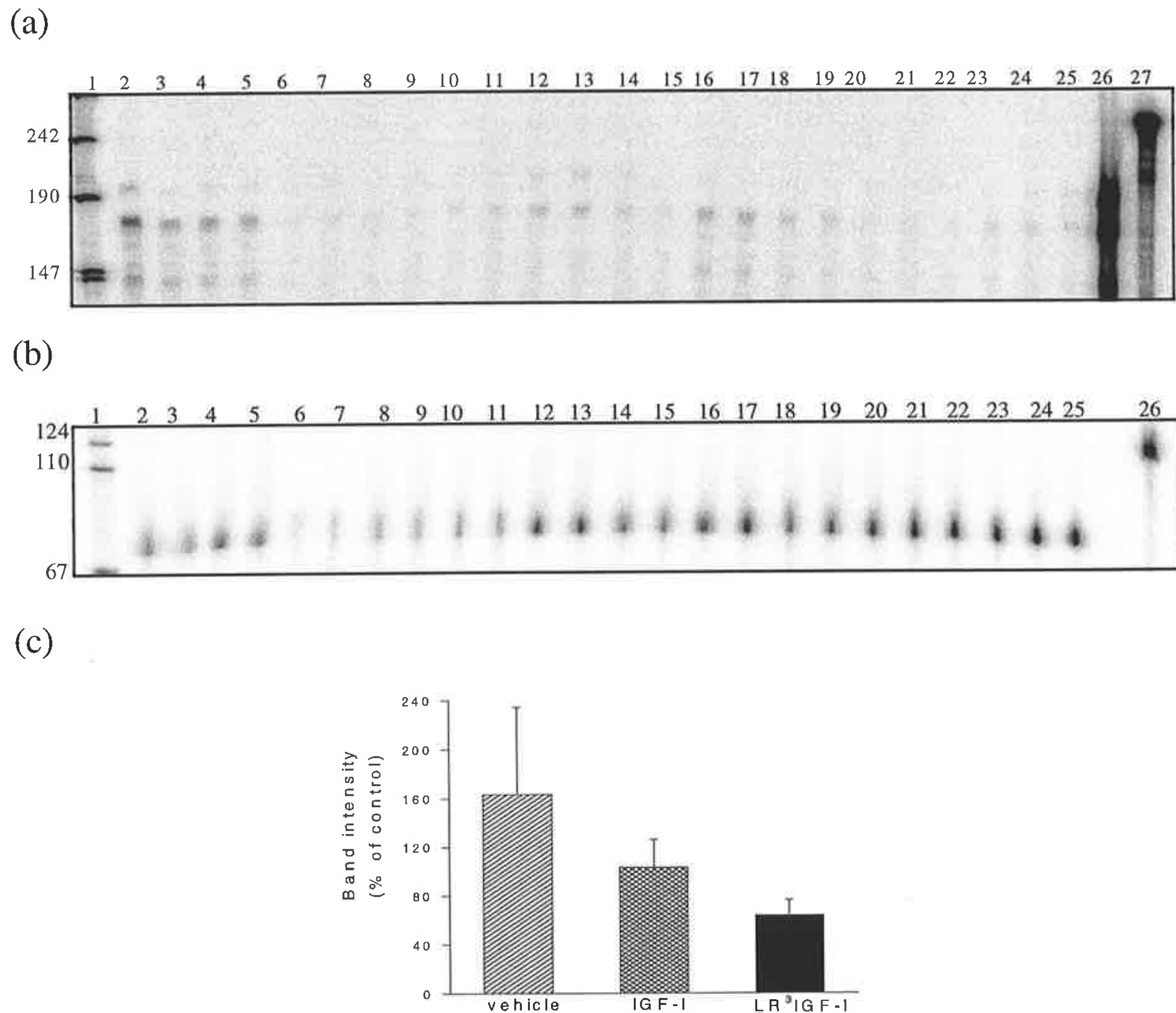


*IGF-I class 2 probe (a) was hybridised with total liver RNA from vehicle (lanes 2 - 9), IGF-I (lanes 10 - 17) or LR^{3}IGF-I treated pigs (lanes 18 - 25). Each sample was analysed in duplicate. Lane 26 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 27 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 6.3b). Significant differences from the vehicle group are denoted by * P < 0.05; ** P < 0.01 and results are expressed as the mean ± SEM for 4 animals per treatment group (b).*

In kidney, neither IGF-I nor LR³IGF-I treatment had a significant effect on the expression of IGF-I *class 1* mRNA (Figure 6.5), although a trend toward decreased levels of IGF-I *class 1* mRNA for both treatment groups was evident when compared to untreated animals (Figure 6.5c).

Figure 6.5:

Effects of IGF-I and LR³IGF-I treatment on IGF-I *class 1* mRNA expression in porcine kidney



IGF-I class 1 probe (a) or 18S RNA probe (b) were hybridised with total kidney RNA from vehicle (lanes 2 - 9), IGF-I (lanes 10 - 17) or LR³IGF-I treated pigs (lanes 18 - 25). Each sample was analysed in duplicate. Lane 26 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 27 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM for 4 animals per treatment group (c).

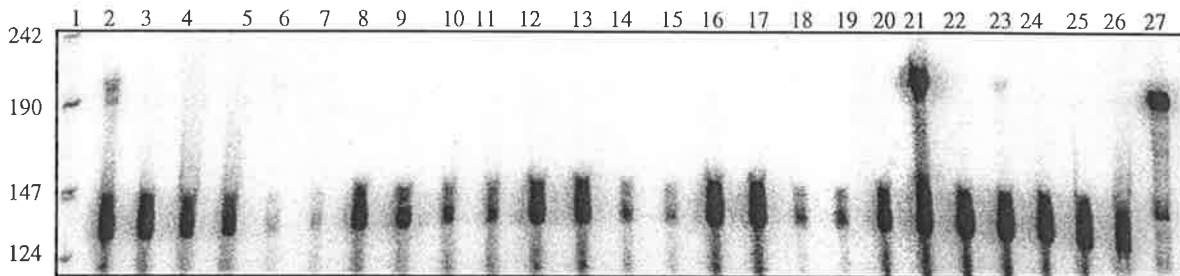
6.3.4 *Effects of IGF-I and LR³IGF-I on IGFBP-3 mRNA expression.*

Treatment with IGF-I or LR³IGF-I had no significant effects on IGFBP-3 mRNA expression in liver (Figure 6.6) or kidney (Figure 6.7), even though LR³IGF-I treatment significantly decreased plasma IGFBP-3 levels as shown by radioimmunoassay in chapter 3 (see Figure 3.3) and western ligand blotting (see Figure 6.2).

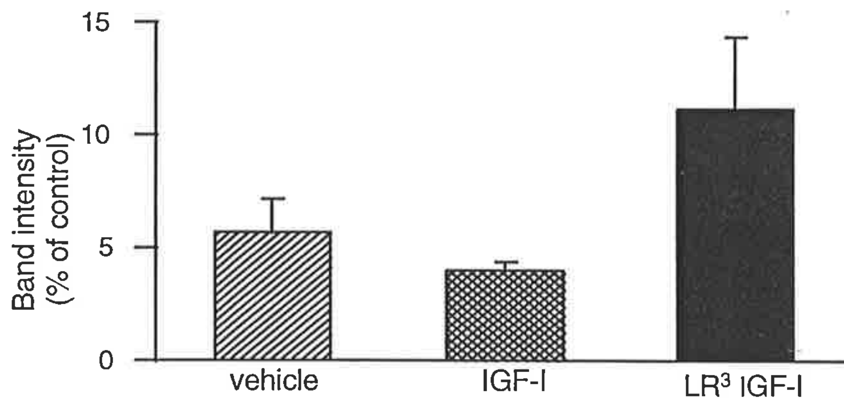
Figure 6.6:

Effects of IGF-I and LR³IGF-I treatment on IGFBP-3 mRNA expression in porcine liver

(a)



(b)

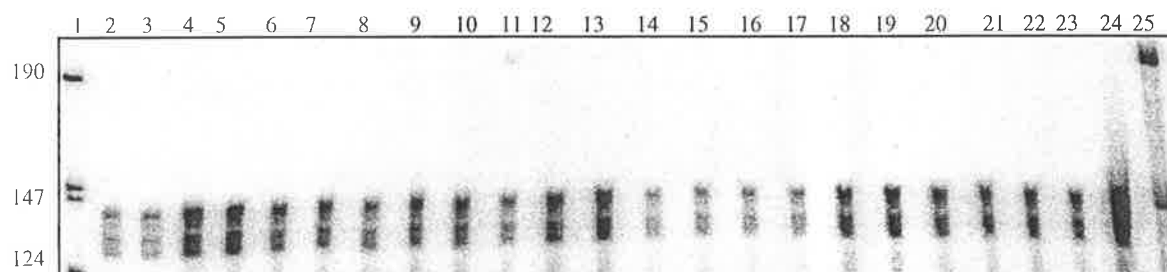


IGFBP-3 probe (a) was hybridised with total liver RNA from vehicle (lanes 2 - 9), IGF-I (lanes 10 - 17) or LR³IGF-I treated pigs (lanes 18 - 25). Each sample was analysed in duplicate. Bands visualised at 190 bases in lanes 2 and 21 represent insufficient digestion with RNase and these samples were not included in the analysis. Lane 26 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 27 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 6.3b). Results are expressed as the mean ± SEM for 4 animals per treatment group (b).

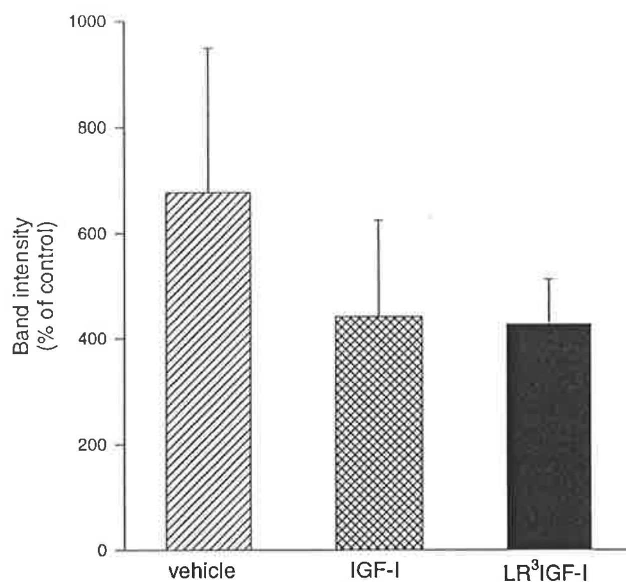
Figure 6.7:

Effects of IGF-I and LR^{3}IGF-I treatment on IGFBP-3 mRNA expression in porcine kidney

(a)



(b)



IGFBP-3 probe (a) was hybridised with total kidney RNA from vehicle (lanes 2 - 7), IGF-I (lanes 8 - 15) or LR^{3}IGF-I treated pigs (lanes 16 - 23). Each sample was analysed in duplicate. Lane 24 represents a control hybridisation to RNA extracted from the liver of GH treated animal, and lane 25 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 6.5b). Results are expressed as the mean ± SEM for 4 animals per treatment group (n = 3 for vehicle) (b).

6.3.5 Summary of results

A summary of the results obtained from the study described in this chapter is given in Table 6.1

Table 6.1:

Effect of IGF-I and LR³IGF-I treatment IGF-I and IGFBP-3 protein and mRNA levels.

	IGF-I	LR³IGF-I
Plasma IGF-I	↑	↓
Plasma IGFBP-3	→	↓
Liver IGF-I class 1 mRNA	→	→
Liver IGF-I class 2 mRNA	↓	↓
Liver IGFBP-3 mRNA	→	→
Kidney IGF-I class 1 mRNA	→	→
Kidney IGFBP-3 mRNA	→	→

Treatment groups are presented in the first row and parameters measured in the first column; → indicates no significant difference from vehicle treated pigs; ↓ indicates significant decrease from vehicle treated pigs; ↑ indicates significant increase from vehicle treated pigs.

6.4 Discussion

The results of the experiment described in chapter 3 show that IGF-I and LR^{3}IGF-I treatment in pigs decreases plasma GH levels and treatment with LR^{3}IGF-I also decreases plasma IGF-I concentrations. Whether this decrease in plasma IGF-I levels is a result of decreased GH-regulated IGF-I mRNA expression is the focus of the present study.

GH treatment increases the expression of IGF-I mRNA in pigs (Brameld *et al.*, 1996; Grant *et al.*, 1991; Coleman *et al.*, 1994; Wolverton *et al.*, 1992) sheep (Hua *et al.*, 1993) and rats (Chow *et al.*, 1994). Experiments described in chapter 5 show that IGF-I gene expression is regulated by GH in liver and kidney. Consequently, the endocrine effects on regulation of IGF-I and IGFBP-3 mRNA expression in these two tissues was investigated.

In liver, neither IGF-I nor LR^{3}IGF-I had a significant effect on IGF-I *class 1* mRNA expression, although a trend towards a reduction in mRNA levels was observed for both treatment groups. IGF-I *class 2* mRNA expression was significantly reduced in liver from IGF-I and LR^{3}IGF-I treated pigs. Results presented in chapter 5 show that in porcine liver, pGH treatment increases the expression of both IGF-I *class 1* and *class 2* mRNA as well as GH receptor mRNA levels (Brameld *et al.,* 1996). The decrease in IGF-I mRNA expression seen in IGF treated animals in the present study may therefore be a result of decreased liver GH receptor mRNA expression. A similar association between IGF-I expression and GH receptor levels has been reported in energy restricted pigs, where a reduction in both IGF-I transcripts and GH receptor mRNA was seen (Weller *et al.*, 1994). In protein restricted rats, plasma IGF-I levels are suppressed and are associated with a decrease in liver GH receptor mRNA (VandeHaar *et al.*, 1991) and IGF-I mRNA expression (Lemozy *et al.*, 1994). Thus there appears to be a direct association between plasma GH levels and liver GH receptor and IGF-I mRNA levels in pigs and rats. Another possibility for the decrease in IGF-I mRNA expression observed in IGF treated pigs is that IGF peptides may be regulating IGF-I mRNA expression through a negative feedback mechanism. The effect of IGF-I treatment on its own expression has been studied in hypophysectomized rats, where the GH effects on IGF-I expression can be controlled. Hypophysectomy (a reduction in plasma GH) in rats results in a decrease in IGF-I mRNA in spleen (Domenè *et al.,* 1994), liver, kidney and several other tissues (Gosteli-Peter *et al.*, 1994). When hypophsectomized rats are treated with IGF-I, plasma IGF-I concentrations are decreased. This is associated with an *increase* in IGF-I mRNA levels in skeletal muscle and white adipose tissue and a *decrease* in liver IGF-I mRNA

levels (Gosteli-Peter *et al.*, 1994). Thus it seems that regulation of IGF-I mRNA expression by IGF-I is complex and differs between tissues.

The differences in mRNA expression between the two classes of IGF-I mRNA in response to IGF treatment indicates that IGF-I *class 2* expression is more sensitive to changes in GH status. This is supported by findings in the previous chapter, where pGH treatment produced a 8 fold increase in expression of IGF-I *class 2* expression while the increase for IGF-I *class 1* expression was only 4 fold. These observations are supported by other studies in pigs (Weller *et al.*, 1994) and sheep (Pell *et al.*, 1993).

GH treatment by subcutaneous injection in normal rats induces hypertrophy of the kidneys (Guler *et al.*, 1988), which is associated with increased levels of kidney IGF-I mRNA (El Nahas *et al.*, 1989). GH exerts its effect on the kidney through systemic and/or locally derived IGF-I (Hirschberg & Kopple, 1988; Hirschberg *et al.*, 1989). In hypophysectomized rats, renal IGF-I mRNA levels are decreased and then restored with GH treatment (Lajara *et al.*, 1989). Studies in the pig show contrasting evidence. IGF-I *class 1* mRNA levels are very low when compared to liver. Treatment with pGH does not affect kidney IGF-I *class 1* mRNA expression (see chapter 5) and treatment with IGF-I or LR^3IGF-I has no significant effect on kidney IGF-I *class 1* mRNA expression, although a trend towards decreased levels of IGF-I *class 1* mRNA are seen in both treatment groups. These findings have also been supported by other studies in the rat, where analysis of total kidney RNA showed no change in the expression of IGF-I mRNA in hypophysectomized, GH or IGF-I treated rats (Gosteli-Peter *et al.*, 1994). Renal IGF-I mRNA is only sensitive to GH status in the collecting ducts but not in other portions of the nephron (Lajara *et al.*, 1989). IGF-I mRNA is also found in the glomerulus and throughout the loop of Henle (Feld & Hirschberg, 1996). It may be possible that RNA analysis on whole kidney tissue (as in the present study and that of Gosteli-Peter *et al.*, 1994) rather than isolated sections of the nephron (by in situ hybridisation; as in the study by Lajara *et al.*, 1989) disguises real IGF-I mRNA changes due to GH status. This may also explain in part the large variability noted in the present study.

The expression of IGFBP-3 mRNA in response to IGF treatment has not previously been studied in the pig. Results discussed in chapter 3 show that LR^3IGF-I treatment decreases plasma IGFBP-3 levels. In the present study, analysis of IGF binding protein profiles confirms the decrease in plasma IGFBP-3 concentrations observed with LR^3IGF-I treatment. This decrease in plasma IGFBP-3 levels is not due to decreased gene expression, since neither IGF-I or LR^3IGF-I affect IGFBP-3 mRNA levels in porcine liver (Figure 6.6) or kidney (Figure 6.7). The most likely cause for the decrease seen in plasma IGFBP-3 levels in LR^3IGF-I treated pigs is post-translational modification of the binding protein by an IGFBP-3 protease.

Low levels of protease activity are found in normal adult sera, with increased protease activity having been reported in pregnancy (Hossenlopp *et al.*, 1990; Gargosky *et al.*, 1990), catabolic states including a post-operative period after major surgery (Cwyfan Hughes *et al.*, 1992), severe illness (Davies *et al.*, 1991), in GH resistant and deficient states (Fielder *et al.*, 1992; Holly *et al.*, 1993), noninsulin-dependent diabetes mellitus (Bang *et al.*, 1994) and malignancies (Muller *et al.*, 1993). Proteolysis of IGFBP-3 has been suggested to be dependent on plasma insulin levels (Berekt *et al.*, 1995). A decrease in IGFBP-3 protease activity has been reported with insulin treatment in patients with insulin dependent diabetes mellitus (Berekt *et al.*, 1995). Whether insulin has a direct or indirect effect on IGFBP-3 protease activity is not clear. LR^3IGF-I treated pigs show reduced plasma insulin levels, average daily gain and feed intake, indicating that they could be in a catabolic state. It is therefore possible that IGFBP-3 proteolysis activity may be increased in these animals, which would account for the decrease in plasma IGFBP-3 but not mRNA levels observed.

It has previously been reported that combination treatment of IGF-I and GH results in an interactive effect by increasing bodyweight gain, plasma IGFBP-3 levels and attenuating the hypoglycaemic effects of IGF-I treatment (Fielder *et al.*, 1996; Kupfer *et al.*, 1993). Results presented in chapter 3 show that combination treatment of IGF-I and pGH increases plasma IGFBP-3 concentrations, but combination treatment does not reverse the negative effects of LR^3IGF-I treatment on ADG, plasma IGF-I, GH or IGFBP-3 when administered for 4 days. Treatment periods in previous studies were considerably longer than those employed in the experiment described in chapter 3, ranging between 14 and 28 days. Whether long term infusion can produce similar results in pigs to those seen in man (Kupfer *et al.*, 1993) and rats (Fielder *et al.*, 1996) is the focus of the next chapter.

CHAPTER 7

COMBINATION TREATMENT OF IGF-I WITH pGH DOES NOT HAVE A SYNERGISTIC EFFECT IN PIGS

7.1 Introduction

Results from the previous series of experiments have shown that treatment with IGF-I does not improve ADG in pigs. This is associated with reduced plasma insulin and GH concentrations and a decrease in liver IGF-I *class 2* mRNA expression. Furthermore, LR^3IGF-I, which is 10 times more potent than IGF-I in promoting cellular proliferation *in vitro* (Francis *et al.*, 1992) and improves growth performance in rats above and beyond that of IGF-I (Tomas *et al.*, 1993), decreases ADG in pigs when compared to the effects of IGF-I. LR^3IGF-I treatment also decreases plasma IGF-I and liver IGF-I *class 2* mRNA expression, plasma IGFBP-3, insulin and GH concentrations (chapters 3 and 6).

GH treatment increases ADG and feed : gain ratio in pigs (Weeden *et al.*, 1993) as well as increasing longitudinal bone growth (Isaksson *et al.*, 1982) and bone wall thickness (Evock *et al.*, 1988). Plasma IGF-I and IGFBP-3 levels have been shown to increase with GH treatment in several species (Hua *et al.*, 1993; Coleman and Etherton, 1991; Glasscock *et al.*, 1991; Domenè *et al.*, 1993). The present study in the pig has shown this to be associated with increased expression of IGF-I and IGFBP-3 mRNA in liver, and IGFBP-3 mRNA in kidney. GH has also been shown to stimulate IGF-I expression in adipose tissue (Wolverton *et al.*, 1992 and semitendinosus but not longissimus dorsi muscle (Brameld *et al.*, 1996).

Combination treatment of IGF-I with GH has been shown to reverse nitrogen wasting, attenuate hypoglycaemia associated with IGF-I and hyperglycaemia associated with GH treatment, and increase IGF-I depleted plasma IGFBP-3 levels in deseased states, for example catabolic humans (Kupfer *et al.*, 1993). In uraemic rats, combination treatment increases body weight gain and longitudinal bone growth above that of either peptide administered alone (Hazel *et al.*, 1994). In hypophysectomized rats, co-administration of IGF-I with GH produces an additive effect on body weight gain (Fielder *et al.*, 1996). Results presented in the short term study discussed in chapter 3 have shown that in pigs, treatment with IGF-I and pGH for 4 days increases plasma IGFBP-3 levels but these are not changed when either peptide is administered alone. In LR^3IGF-I treated pigs, there does not appear to be a similar synergistic effect on plasma IGFBP-3 levels. LR^3IGF-I treatment results in decreased plasma IGFBP-3 levels, which remain suppressed when pGH is added to the treatment regime. It could be argued though that there is a trend towards returning plasma IGFBP-3 to normal levels. In addition, plasma IGF-I levels are also reduced in both these treatment groups. Previous studies where IGF-I had been administered in combination with GH between 14 to

28 days showed additive effects on body weight gain and plasma IGFBP-3 levels (Fielder *et al.*, 1996; Kupfer *et al.*, 1993). In the study presented in chapter 3, the treatment period was for only 4 days. This may have been an insufficient time period to show synergistic effects between the two treatments.

The aim of the present experiment is to determine if co-administration of pGH with either IGF-I or LR^3IGF-I for 14 days does have a synergistic effect in pigs by determining if co-administration with pGH can eliminate the reduction in IGF-I protein and mRNA levels as well as IGFBP-3 plasma levels in pigs treated only with IGF-I or LR^3IGF-I.

7.2 Materials and Methods

7.2.1 Peptides and plasmids

Recombinant human IGF-I and LR^3IGF-I were supplied by GroPep Pty. Ltd., Adelaide, SA, Australia. Porcine GH was supplied by Bresatec Pty Ltd., Adelaide, Australia. Purified porcine IGFBP-3 was purified in this laboratory by Dr Paul Walton. Plasmids pRP1 and pRP2 were a gift from Dr Stewart Gilmour. Plasmid p131BP3 was generated as described in chapter 4 section 4.2.17. pT718SRNA was purchased from Ambion Inc., Austin, Texas, USA.

7.2.2 Enzymes

Restriction enzymes Sal I and Bam HI were obtained from Progen Industries Ltd., Darra, Australia. Proteinase K, RQ1 RNase-free DNase and rRNasin were obtained from Promega Corporation, Madison, WI, USA. RNase A and RNase T1 were obtained from Sigma Chemical Company, St. Louis, MO, USA.

7.2.3 Radionucleotides

α-^{35}S-dATP, α-^{32}P-UTP and γ-^{32}P-dATP were obtained from Bresatec Ltd, Adelaide, Australia. Na-I^{125} was obtained from Amersham Australia Pty Ltd., NSW, Australia.

7.2.4 Chemicals

All chemicals were of analytical reagent grade, and were obtained from Sigma Chemical Company, St. Louis, MO, USA, Merk Pty Ltd, Kilsyth, Victoria, Australia or Ajax Chemicals, Auburn, NSW, Australia.

7.2.5 Animals and animal maintenance

Pigs were obtained and housed at the Victorian Institute of Animal Science, Werribee, Victoria, Australia. The animals were individually penned in metabolism cages throughout the experiment and were fed 110g/kg bodyweight per day.

7.2.6 Experimental design

Study 1: Sixteen female cross bred pigs (55 kg) were grouped on liveweight and within each group were randomly allocated in a 2 x 2 factorial experimental design into 4 treatment groups with the respective factors being growth factor (vehicle or IGF-I) and growth hormone (saline or pGH). Alzet miniosmotic pumps (Alza, Palo Alto, CA, USA) were subcutaneously inserted into the neck region. IGF-I was infused at a rate of 180 µg/kg/day and growth hormone was administered at a dose of 30 µg/kg/day by daily intra muscular injection. Animals were treated for 14 days. Vehicle treatments were acetic acid (100 mmol/l) for IGF-I and NaCl (150 mmol/l) for pGH. Blood samples were taken by venipuncture on days 0, 4, 8 and 15. On the seventh treatment day, urinary catheters were inserted. At the completion of the treatment period, animals were sacrificed, pituitary and tissue samples were taken from the left anterior lobe of the liver and left kidney, snap frozen in liquid nitrogen and stored at -80°C until analysed.

Study 2: This study was identical in design to study 1 except that LR^3IGF-I was the administered growth factor.

The studies was approved by the Victorian Institute of Animal Science Animal Experimentation Ethics Committee and the Animal Ethics Committee from the University of Adelaide.

7.2.7 Determination of average daily gain

Pigs were weighed after 3, 5, 7, 8 and 15 days of treatment on commercial livestock scales that had been incorporated into a metabolism cage.

7.2.8 Determination of plasma IGF-I concentration

Iodination of human IGF-I for use in IGF-I radioimmunoassay was performed as described in chapter 3 section 3.2.6.

IGF peptides were separated from the IGF binding proteins by acid gel-filtration as described in chapter 3 section 3.2.7.1 and plasma IGF-I concentrations were determined by radioimmunoassay as described in chapter 3 section 3.2.7.2.

7.2.9 Determination of plasma GH concentration

Plasma GH concentration in blood samples and pituitary homogenate were determined by GH radioimmunoassay as described in chapter 3, section 3.2.10.

7.2.10 Determination of pituitary GH content

Whole pituitaries were homogenised using a hand held glass homogeniser in 20 µl/mg tissue homogenisation buffer (25 mM Tris; 150 mM NaCl, pH 7.6). A 200 µl aliquot of pituitary extract was diluted with 1.8 ml homogenisation buffer, centrifuged at 1500 x *g* for 30 minutes at 4°C and the supernatant recovered. The supernatant was diluted 1 : 3333 and a 200 µl aliquot used in a GH radioimmunoassay as described in chapter 3 section 3.2.10.

7.2.11 Determination of plasma IGFBP-3 concentration

Plasma IGFBP-3 concentration were determined by radioimmunoassay as described in chapter 3 section 3.2.8.

7.2.12 Analysis of IGF-I class 1, IGF-I class 2 and IGFBP-3 gene expression

Total RNA was extracted from liver and kidney as described in chapter 4 section 4.2.18 and the integrity of the RNA was checked by running aliquots on 1 % agarose gels as described in section 4.2.10. Radioactive molecular weight markers were generated as described in chapter 4 section 4.2.19. Riboprobes were generated as described in chapter 4 section 4.2.20 for the detection of IGF-I *class 1* and IGF-I *class* 2 mRNA, section 4.2.21 for the detection of IGFBP-3 mRNA and section 4.2.22 for the detection of 18 S ribosomal RNA expression. RNase protection assays were carried out as described in section 4.2.23 with the exception that 50 μg of total RNA was used for detection of IGF-I *class 1*, *class* 2 and IGFBP-3 mRNA and 10 μg of total RNA was used for the detection of 18S ribosomal RNA.

7.2.13 Statistical analysis

All data was analysed using SigmaStat™ statistical software version 1.0 (Jandel Scientific Software, USA). All data was analysed using a 2 x 2 factorial design two way analysis of variance with the respective factors being growth factor (vehicle or IGF-I) and growth hormone (vehicle or pGH). Significant differences were determined using Bonferroni's method of comparison. Data are expressed as the mean ± SEM with n = 4.

7.3 Results

7.3.1 Growth performance of pigs treated with LR^{3}IGF-I alone and in combination with pGH

This study could not be completed. Animals treated with LR^{3}IGF-I alone or in combination with pGH lost their appetite. Two animals (one from the LR^{3}IGF-I group, the other from the LR^{3}IGF-I + pGH group) had to be euthanised after 7 and 9 days of treatment. Another LR^{3}IGF-I treated animal was found dead in its pen on day 10 of treatment.

7.3.2 Growth performance of pigs treated with IGF-I alone and in combination with pGH

Average daily weight gain (ADG) and feed intake during 14 days of treatment are shown in Table 7.1. Statistical analysis of the data show that ADG was increased by pGH throughout the treatment period. IGF-I treatment had no effect on ADG at any of the time points measured throughout the treatment period. After 7 days of treatment, the combination treatment of IGF-I + pGH had a synergistic effect on ADG. This increase in ADG did not continue for the remainder of the treatment period. Feed intake or feed : gain ratio was not affected by any of the treatment groups.

7.3.3 Plasma IGF-I concentrations

Plasma IGF-I concentrations were measured in blood samples taken on day 0, 4, 8 and 15 of the treatment period. (Table 7.2). Animals treated with IGF-I showed a significant increase in plasma IGF-I levels throughout the treatment period. GH treatment had no effect on plasma IGF-I levels except on the last day of treatment, where animals receiving pGH showed increased levels of plasma IGF-I when compared to those not receiving pGH. There were no interactive effects between IGF-I and pGH treatment on plasma IGF-I levels.

Table 7.1:

Effect of vehicle or IGF-I infusion alone or in combination with porcine GH injection on average daily gain (ADG), feed intake and feed : gain ratio over the treatment period

	GH			IGF			Significance[1]		
	control	pGH	*sed*[2]	vehicle	IGF-I	*sed*[3]	G	I	G x I
ADG (g):									
Day 0 - 3	975	1408*	*183.2*	1125	1258	*183.2*	0.036	0.481	0.197
Day 0 - 5	925	1235*	*96*	1050	1150	*96*	0.007	0.544	0.102
Day 0 - 7	854	1189*	*86.4*	1011	1032	*86.4*	0.002	0.808	0.039
Day 0 - 8	800	1122*	*86.2*	953	969	*86.2*	0.003	0.859	0.255
Day 0 - 15	442	744*	*78.3*	636	550	*78.3*	0.003	0.295	0.093
Feed Intake (g/kg$^{0.75}$/day)	101.5	108.1	*4.05*	111.0	98.6	*4.05*	0.29	0.06	0.47
Feed : gain ratio	5.79	2.67	*0.548*	3.27	5.18	*0.548*	0.067	0.242	0.219

*Significant differences from the vehicle group are denoted by * with the appropriate P values given in the table; [1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I) (note an interactive effect after 7 days of treatment); [2]- standard error of the difference of means for GH (G), n = 8; [3]- standard error of the difference of means for growth factor (I), n = 8.*

Table 7.2:

Effect of IGF-I, pGH or combination IGF-I + pGH treatment on plasma IGF-I concentration

	GH			IGF			Significance[1]		
	control	pGH	*sed*[2]	vehicle	IGF-I	*sed*[3]	G	I	G x I
Plasma IGF-I (ng/ml):									
Day 0	244.6	208.5	*11.2*	214.1	239	*11.2*	0.277	0.448	0.814
Day 4	388.4	490.6	*18.7*	324.9	554.1*	*18.7*	0.08	0.001	0.82
Day 8	305.5	399	*18.9*	271.1	433.4*	*18.9*	0.11	0.01	0.43
Day 15	239.8	415.7*	*29.6*	268.8	386.6*	*29.6*	0.002	0.02	0.44

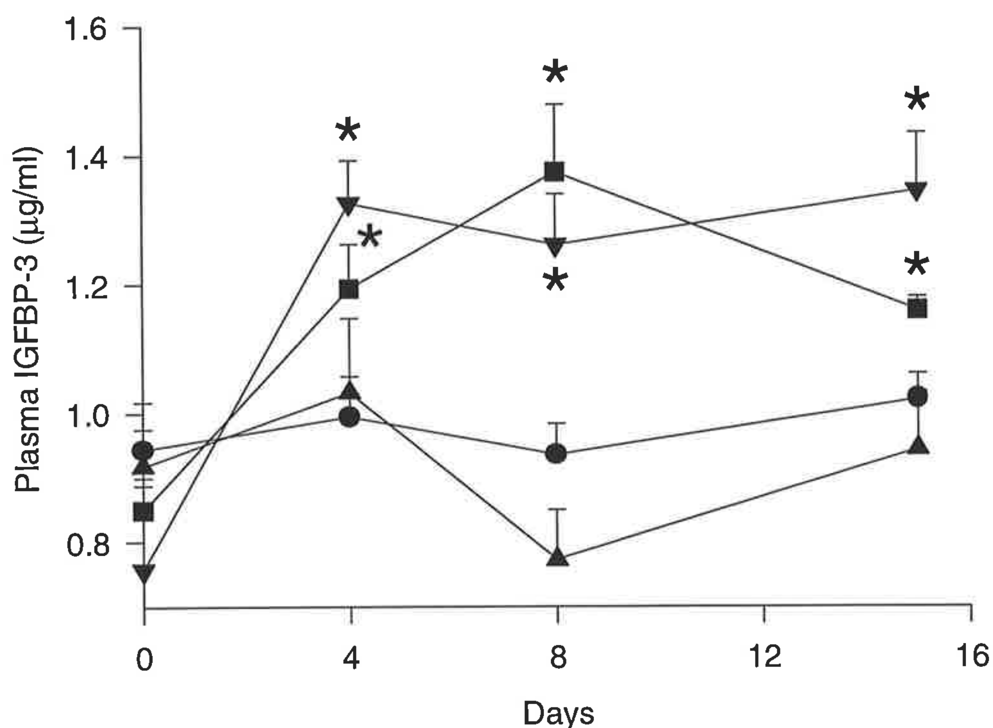
*Significant differences from the vehicle group are denoted by * with the appropriate P values given in the table; [1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I); [2]- standard error of the difference of means for GH (G), n = 8; [3]- standard error of the difference of means for growth factor (I), n = 8.*

7.3.4 Plasma IGFBP-3 concentrations

Treatment with IGF-I for 14 days had no effect on plasma IGFBP-3 concentrations. GH treatment, whether alone or in combination with IGF-I significantly increased plasma IGFBP-3 levels throughout the treatment period (Figure 7.1).

Figure 7.1:

Effect of vehicle (●), pGH (■), IGF-I (▲) or IGF-I + pGH (▼) treatment on plasma IGFBP-3 concentrations



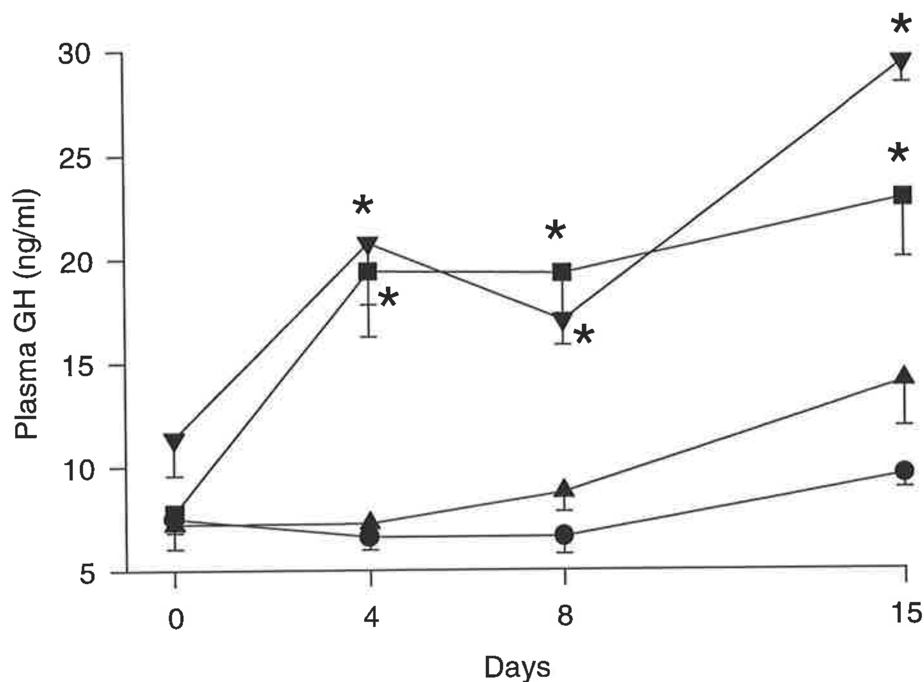
*Results are expressed as the mean ± SEM for 4 animals in each treatment group. Significant differences are denoted by * indicating a significant increase in plasma IGFBP-3 compared to the vehicle treated group ($p < 0.05$).*

7.3.5 Plasma GH concentrations

Plasma GH concentrations were measured in blood samples obtained on days 0, 4, 8 and 15. Treatment with GH significantly increased plasma GH levels throughout the treatment period, while IGF-I alone had no effect on plasma GH levels (Figure 7.2).

Figure 7.2:

Effect of vehicle (●), pGH (■), IGF-I (▲) or IGF-I + pGH (▼) treatment on plasma GH concentrations



*Results are expressed as the mean ± SEM for 4 animals in each treatment group. Significant differences are denoted by * indicating a significant increase in plasma GH compared to the vehicle treated group (P < 0.05).*

7.3.6 Effects of IGF-I treatment alone and in combination with pGH on pituitary weight and total pituitary GH content.

Total pituitary weight was significantly increased by pGH treatment but was not affected by IGF-I treatment. Pituitary GH content was not altered by any of the treatments (Table 7.3).

Table 7.3:

Effect of IGF-I, pGH or combination IGF-I + pGH treatment on total pituitary weight and GH content.

	GH			IGF			Significance[1]		
	control	pGH	*sed*[2]	vehicle	IGF-I	*sed*[3]	G	I	G x I
Pituitary weight (mg)	247.7	280.0*	*9.59*	260.6	267.1	*9.59*	0.04	0.65	0.81
Pituitary GH content (μg/mg of tissue)	19.7	20.2	*1.25*	21.1	18.8	*1.25*	0.77	0.24	0.14

*Significant differences from the vehicle group are denoted by * with the appropriate P values given in the table; [1]- significance of difference of pGH (G), growth factor (I) or interaction (G x I); [2]- standard error of the difference of means for GH (G), n = 7; [3]- standard error of the difference of means for growth factor (I), n = 7.*

7.3.7 Effect of IGF-I treatment alone and in combination with pGH on IGF-I class 1 and class 2 mRNA expression.

IGF-I *class 1* gene expression is represented by two protected fragments of 200 and 170 bases. A autoradiogram from RNase protection assays to detect the expression of IGF-I *class 1* mRNA in liver is shown in Figure 7.3. Treatment with IGF-I significantly decreased expression of IGF-I *class 1* mRNA ($P < 0.003$). When IGF-I was administered in combination with pGH, IGF-I *class 1* mRNA levels remained suppressed. Fourteen days of treatment with pGH had no effect on the expression of IGF-I *class 1* mRNA expression.

IGF-I *class* 2 mRNA expression is represented by one protected fragment of 190 bases (Figure 7.4). Treatment with pGH significantly increased the expression of IGF-I *class 2* mRNA ($P < 0.0002$), while IGF-I treatment significantly decreased the expression of *IGF-I class* 2 mRNA ($P < 0.0001$). Co-administration of pGH with IGF-I returned expression of IGF-I *class* 2 mRNA to a level comparable to those of the control group.

In kidney, none of the treatments had a significant effect on the expression of IGF-I *class 1* mRNA (Figure 7.5)

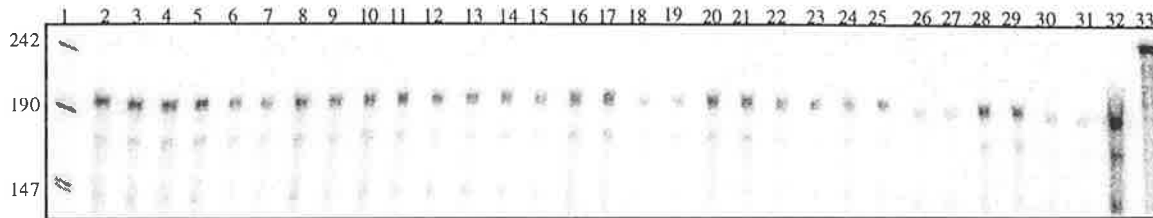
7.3.8 Effect of IGF-I treatment alone and in combination with pGH on IGFBP-3 mRNA expression.

Treatment with IGF-I alone or in combination with pGH had no significant effect on IGFBP-3 mRNA expression in liver (Figure 7.6) or kidney (Figure 7.7).

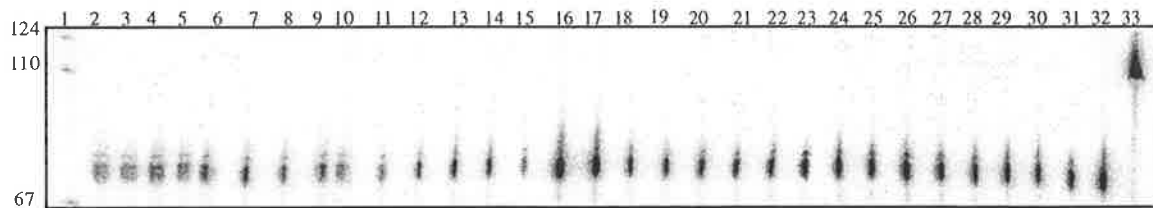
Figure 7.3:

Effects of IGF-I treatment alone or in combination with pGH on IGF-I *class 1* mRNA expression in porcine liver

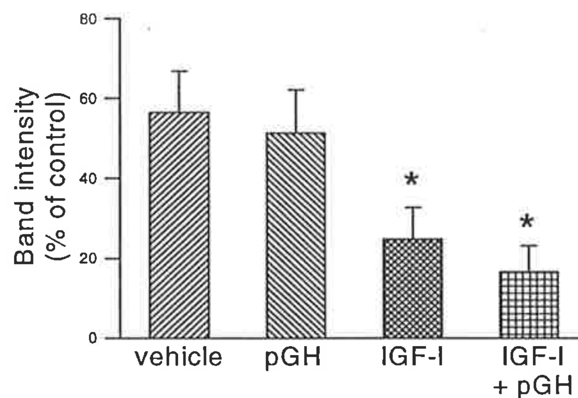
(a)



(b)



(c)

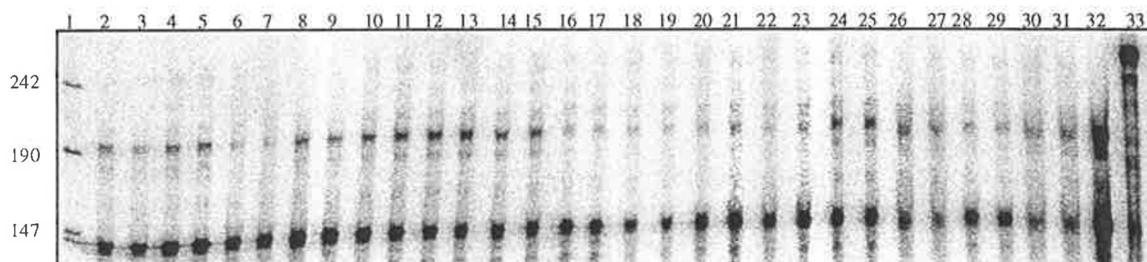


*IGF-I class 1 probe (a) or 18S RNA probe (b) were hybridised with total liver RNA from vehicle (lanes 2-9), pGH (lanes 10-15), IGF-I (lanes 16-23) or IGF-I + pGH treated pigs (lanes 24-31). Each sample was analysed in duplicate. Lane 32 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 33 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. * represents a significant decrease in IGF-I class 1 mRNA when compared to animals not receiving IGF-I ($P < 0.003$). Band intensities are expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe. Results are expressed as the mean ± SEM for 4 animals per treatment group ($n = 3$ for pGH group) (c).*

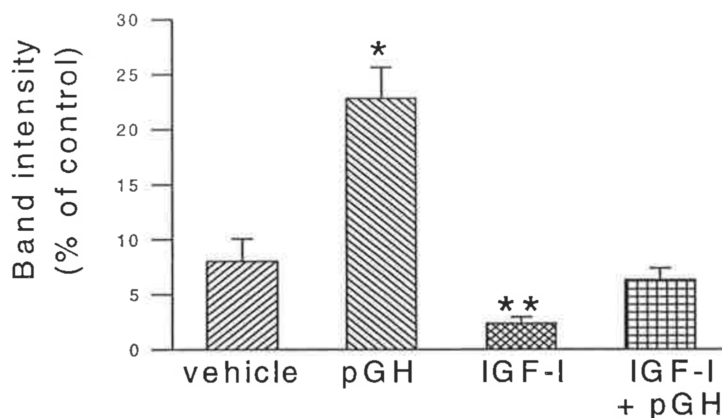
Figure 7.4:

Effects of IGF-I treatment alone or in combination with pGH on IGF-I *class 2* mRNA expression in porcine liver

(a)



(b)

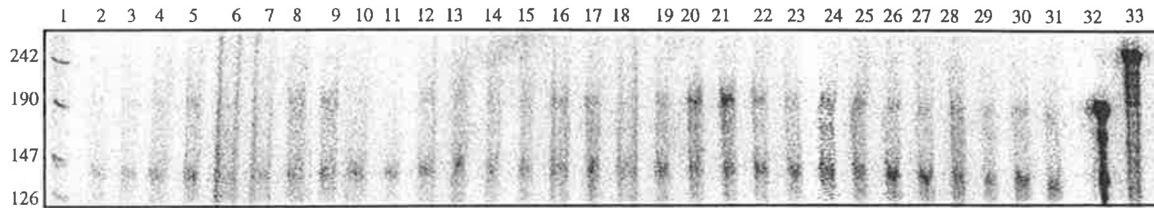


*IGF-I class 2 probe (a) was hybridised with total liver RNA from vehicle (lanes 2-9), pGH (lanes 10-15), IGF-I (lanes 16-23) or IGF-I + pGH treated pigs (lanes 24-31). Each sample was analysed in duplicate. Lane 32 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 33 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities were expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 7.3b). * represents a significant increase in IGF-I class 2 mRNA when compared to animals not receiving pGH, ($P < 0.0002$) and ** represents a significant decrease in IGF-I class 2 mRNA when compared to animals not receiving IGF-I ($P < 0.0001$) Results are expressed as the mean ± SEM for 4 animals per treatment group ($n = 3$ for pGH group) (b)*

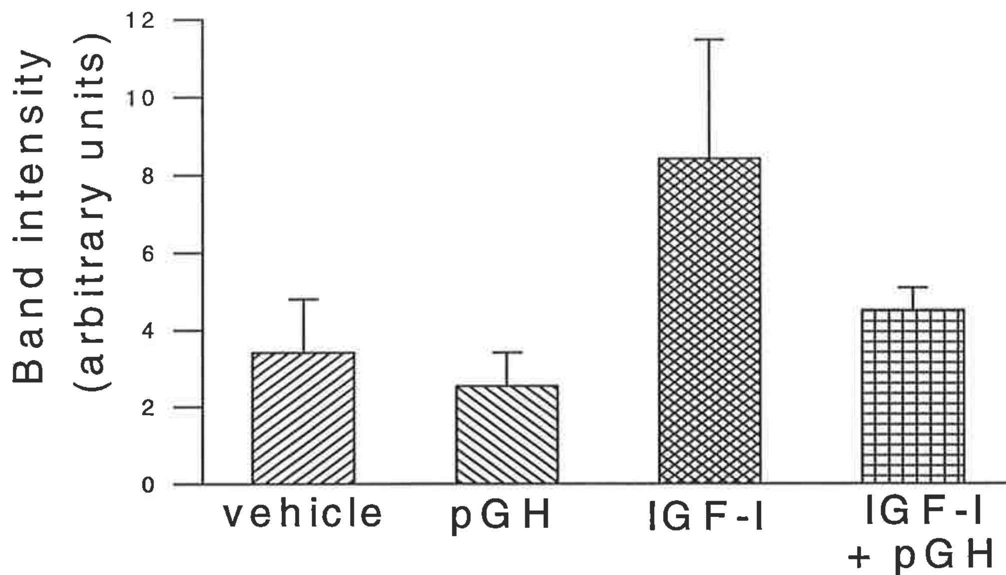
Figure 7.5:

Effects of IGF-I treatment alone or in combination with pGH on IGF-I *class 1* mRNA expression in porcine kidney

(a)



(b)

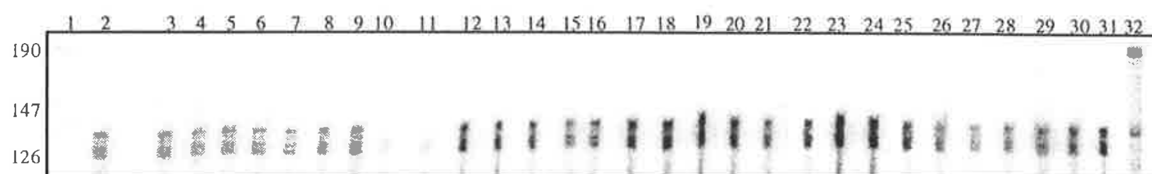


IGF-I class 1 probe (a) was hybridised with total liver RNA from vehicle (lanes 2-9), pGH (lanes 10-15), IGF-I (lanes 16-23) or IGF-I + pGH treated pigs (lanes 24-31). Each sample was analysed in duplicate. Lane 32 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 33 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Results are expressed as the mean ± SEM for 4 animals per treatment group (n = 3 for pGH group) (b). Band intensities were unable to be normalised to 18S controls due to insufficient sample.

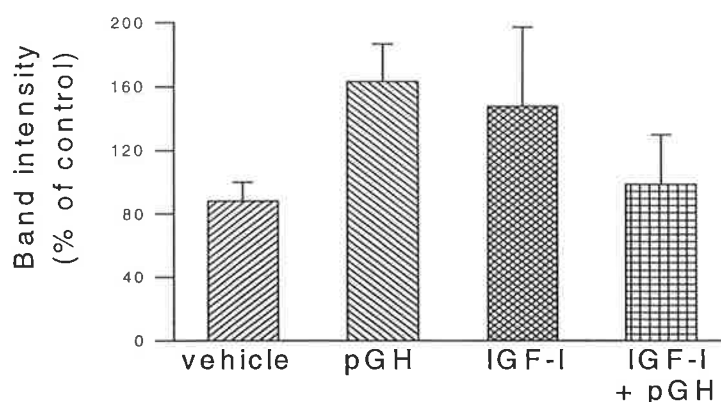
Figure 7.6:

Effects of IGF-I treatment alone or in combination with pGH on IGFBP-3 mRNA expression in porcine liver

(a)



(b)

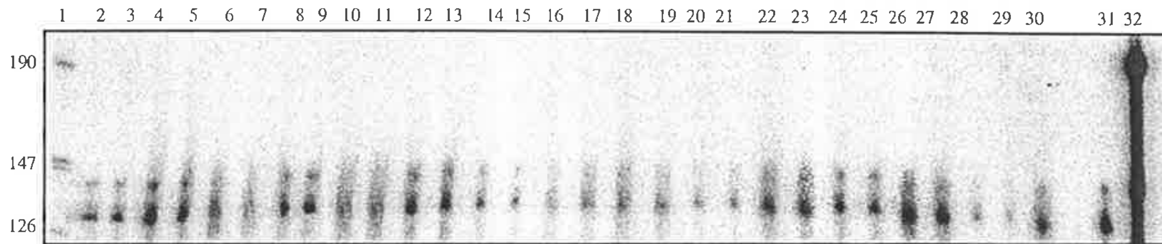


IGFBP-3 probe (a) was hybridised with total liver RNA from vehicle (lanes 2-9), pGH (lanes 10-14), IGF-I (lanes 15-22) or IGF-I + pGH treated pigs (lanes 23-30). Each sample was analysed in duplicate (except lane 14). Lane 31 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 32 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Band intensities were expressed as a percentage of the intensity obtained from hybridising the same RNA samples with a 18S RNA probe (Figure 7.3b). Results are expressed as the mean ± SEM for 4 animals per treatment group (n = 3 for pGH group) (b).

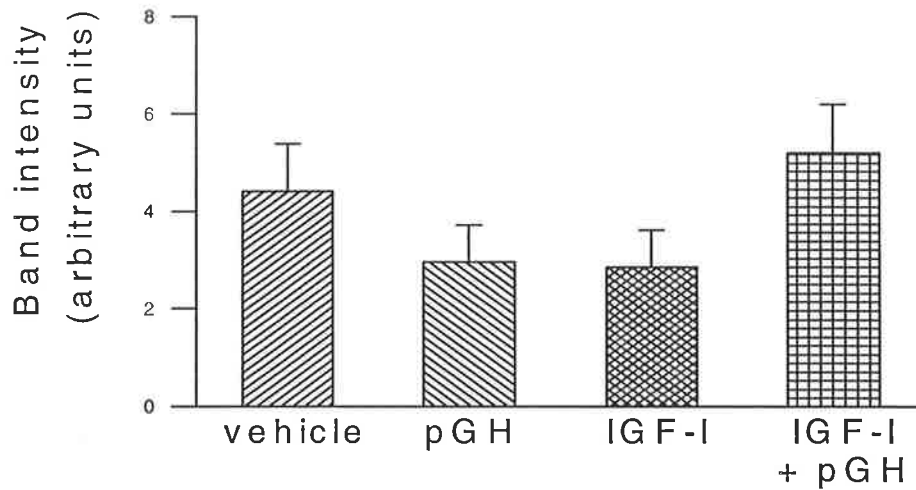
Figure 7.7:

Effects of IGF-I treatment alone or in combination with pGH on IGFBP-3 mRNA expression in porcine kidney

(a)



(b)



IGFBP-3 probe (a) was hybridised with total kidney RNA from vehicle (lanes 2-9), pGH (lanes 10-15), IGF-I (lanes 16-23) or IGF-I + pGH treated pigs (lanes 24-30). Each sample was analysed in duplicate (except lane 30). Lane 31 represents a control hybridisation to RNA extracted from the liver of pGH treated animals, and lane 32 represents the full length riboprobe. DNA molecular weight markers are indicated (in bases) in lane 1. Results are expressed as the mean ± SEM for 4 animals per treatment group (n = 3 for pGH group) (b). Band intensities were unable to be normalised to 18S controls due to insufficient sample.

7.3.9 Summary of results

A summary of the results obtained from the study described in this chapter is given in Table 7.4

Table 7.4:

Effect of IGF-I treatment alone and in combination with pGH on growth performance, plasma metabolites and IGF-I and IGFBP-3 mRNA expression.

	IGF-I	pGH	IGF-I + pGH
Pituitary weight	→	↑	→
Pituitary GH content	→	→	→
ADG	→	↑	↑ (0-7 days)
Feed intake	→	→	→
Plasma IGF-I	↑	↑ (on last day)	↑[1]
Plasma IGFBP-3	→	↑	↑
Plasma GH	→	↑	↑[2]
Liver IGF-I class 1 mRNA	↓	→	↓[1]
Liver IGF-I class 2 mRNA	↓	↑	→
Liver IGFBP-3 mRNA	→	→	→
Kidney IGF-I class 1 mRNA	→	→	→
Kidney IGFBP-3 mRNA	→	→	→

Treatment groups are presented in the first row and parameters measured in the first column; → indicates no significant difference from vehicle treated pigs; ↓ indicates significant decrease from vehicle treated pigs; ↑ indicates significant increase from vehicle treated pigs; [1] indicates an effect only due to IGF-I treatment; [2] indicates an effect only due to pGH treatment.

7.4 Discussion

In the present study, 14 days of treatment with LR^3IGF-I alone or in combination with pGH was fatal in some pigs. All animals receiving this treatment exhibited reduced appetites and may have died due to hypoglycaemia induced by fasting. These fatal effects have also been observed in two previous studies (Walton, unpublished observations). In the first study, pigs were treated with LR^3IGF-I at a dose of 450 μg/kg/day for 14 days. Two animals died on day 5 and 7 of treatment. Plasma glucose concentrations were measured at the completion of the trial in the surviving animal, and were found to be within the normal range. Plasma glucose levels were also unchanged in a separate experiment where animals received either 18, 54 or 180 μg/kg/day LR^3IGF-I for 14 days. In this experiment, no animals died, although ADG but not feed intake was significantly reduced with LR^3IGF-I treatment. An explanation for the mortality rate in the present study is that LR^3IGF-I treatment in addition to acting on the GH/IGF-I axis, does cause metabolically toxic effects in pigs that are not symptomatic in all animals. Indeed, blood glucose levels measured in those pigs prior to euthanasia indicated that plasma glucose where outside the normal range, being either suppressed (1.33 mmol/l for one LR^3IGF-I treated and 2.28 mmol/l for one LR^3IGF-I + pGH treated pig) or elevated (10.07 mmol/l for one LR^3IGF-I treated pig). In addition, these animals presented with muscular twitching and became comatosed prior to death, all indications that metabolic toxicity may be the cause of death.

The synergistic actions of simultaneous treatment with IGF-I and GH have been demonstrated in uraemic rats (Hazel *et al.*, 1994), calorie restricted healthy male volunteers (Kupfer *et al.,* 1993) and hypophysectomized rats (Fielder *et al.*, 1996). In the present study, the combination treatment of IGF-I and pGH did not have added beneficial effects on plasma hormone levels or their mRNA expression when compared to pGH treatment alone. The only exception to this was that in the first week of treatment, animals receiving both peptides showed an increase in ADG that was due to an interactive effect between IGF-I and pGH treatment. This increase in ADG was not maintained for the remainder of the treatment period. This may be due to the fact that at this time, animals were fitted with urinary catheters. The lack of synergistic actions of simultaneous treatment with IGF peptides and pGH observed in the present experiment reinforce those presented in chapter 3, where ADG, feed intake and feed : gain ratio were also not improved when pigs were treated with IGF-I or LR^3IGF-I in combination with pGH for 4 days.

As, expected, treatment with IGF-I alone, or in combination with pGH resulted in elevated levels of plasma IGF-I. The elevation in plasma IGF-I is entirely due to exogenous administration of IGF-I, since animals treated with IGF-I show a decrease in the expression of IGF-I *class 1* and IGF-I *class 2* mRNA in liver. Co-administration of pGH with IGF-I may have had a compensatory effect on IGF-I *class 2* mRNA expression, since the levels of this transcript in liver were not different to control. In contrast, this compensatory effect was not evident with IGF-I *class 1* mRNA expression, which remained suppressed in animals receiving the combination treatment. This is consistent with the findings in the present experiment where pGH treatment alone had no effect on expression of IGF-I *class 1* mRNA in liver.

In the present experiment, plasma IGF-I levels were increased with pGH treatment but only in blood samples taken on the last day of treatment. Thus it appears that longer term treatment with a moderate dose of pGH (30 μg/kg/day) as used in the present experiment can elevate plasma IGF-I levels. In order to increase circulating IGF-I levels during short term treatment, pGH doses of at least 50 μg/kg/day are required to elicit a response (Chung *et al.*, 1985).

The increase in plasma IGF-I levels with pGH treatment in the present study is associated with an increase in IGF-I *class 2* but not IGF-I *class 1* mRNA expression in the liver. Results obtained from the experiment described in chapter 5 show that both IGF-I transcripts are elevated with pGH treatment (at a dose of 70 μg/kg/day) and IGF-I *class 1* mRNA expression is less sensitive to pGH treatment. This appears to also be the case in the present study where IGF-I *class 1* gene expression was unaltered by pGH treatment when administered at the lower dose used in the present study.

Plasma IGFBP-3 levels were unaffected by IGF-I treatment, whereas pGH treatment alone and in combination with IGF-I significantly increased plasma IGFBP-3. This is consistent with GH being the primary regulator of IGFBP-3 expression in the pig. However, the increase in plasma IGFBP-3 levels was not due to increased transcription of the IGFBP-3 gene, since IGFBP-3 mRNA levels were not altered in liver or kidney with any of the treatment groups. Growth hormone regulates the expression of the acid-labile subunit (Zapf *et al.*, 1989) which forms a ternary complex with IGF-I or IGF-II and IGFBP-3 (Baxter *et al.*, 1989). It is possible that in the present study, pGH treatment increased the expression of the acid-labile subunit which allows more IGFBP-3 to be bound in this complex, thereby increasing the half life and elevating plasma IGFBP-3 levels without any alteration in IGFBP-3 gene expression. It should be noted though that findings from the experiments described in

chapter 5 show that a higher dose of pGH (70 μg/kg/day) does increase the expression of IGFBP-3 mRNA in liver and kidney.

As expected, treatment with pGH significantly increased plasma GH levels, whereas in this study, IGF-I treatment had no effect on plasma GH levels. The manner in which plasma GH levels were determined in the present experiment (one sample every 4 days) are not the ideal method for establishing plasma GH levels. GH exhibits a pulsatile pattern of expression and should be measured at very short time intervals (at least every 10 minutes). Pulsatile analysis of GH secretion was measured in the experiments described in chapter 3 and showed that IGF-I treatment decreased plasma GH levels. This is probably a result of negative feedback inhibition on GH expression and/or secretion from the pituitary. IGF-I has been shown to inhibit GH secretion and mRNA expression in rat pituitary cell culture (Yamashita and Melmed, 1986). In the present study, IGF-I treatment did not affect total GH content in the pituitary, suggesting that IGF-I acts to inhibit GH expression rather than its release from the pituitary. The effect of pGH on pituitary weight has been previously established (Andres *et al.,* 1993). GH treatment increases posterior and anterior pituitary weights in a dose dependent manner. Length of treatment also appears to play a role in the effects of pGH on pituitary weight. Treatment for 4 days at 30 μg/kg/day had no significant effect on pituitary weight, whereas treatment at the same dose for two weeks significantly increased pituitary weight.

In the present study, there was only a transient synergistic effect observed in ADG after 7 days of IGF-I + pGH treatment. Analysis of plasma metabolites and their gene expression indicates that combination treatment of IGF-I with pGH does not produce a synergistic effect in pigs. This is largely due to the negative effects IGF-I treatment has on IGF-I gene transcription. Although the combination treatment does result in increased levels of plasma IGF-I, IGFBP-3 and GH, these results are not due to an interactive effect between the two peptides, but rather a compensatory effect of pGH administration.

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

Manipulation of growth characteristics in commercially relevant species either by transgenesis, breeding or treatment with growth enhancers is an important and ongoing area of research. Many of the actions of GH are mediated via IGF-I, and GH treatment has been successful in improving the growth performance of pigs. These encouraging findings prompted the research into the effects of IGF-I and IGF-I analogue treatment on growth performance in pigs as an alternative or in addition to pGH. The results were clearly disappointing. The growth rates were either not different to control animals when treated with IGF-I, or were reduced and associated with increased mortality when the potent IGF-I analogue LR^3IGF-I was used. Furthermore, a combination of IGF peptides and GH was ineffective in enhancing growth performance, or even in reversing the negative effects of the IGF peptides. In contrast, IGF-I and LR^3IGF-I increase body weight gain in rats (Tomas *et al.*, 1993). These conflicting results may be caused by a number of different factors acting alone, or more likely, in combination. Firstly, IGF-I administration has been shown to cause hypoglycaemia in several species (Guler *et al.*, 1987; Zapf *et al.*, 1986; Walton *et al.*, 1989b), resulting in decreased glucose availability. This decrease in glucose metabolism may be a contributing factor to the poor growth performance of pigs treated with IGF peptides. Secondly, IGF peptides may also have additional toxic effects in pigs unrelated to their insulin-like actions. Thirdly, the IGF peptides may be acting to reduce GH secretion or synthesis and that this reduction in plasma GH may affect endogenous plasma IGF-I and IGFBP-3 levels. The consequence of this is a reduction in growth performance. It is this third hypothesis that is addressed in the current study and therefore the aims of this thesis were to determine:

- the effects of IGF-I or LR^3IGF-I infusion on growth performance and components of the GH/IGF-I axis.
- in which porcine tissues IGF-I and IGFBP-3 are regulated by GH
- the effects of IGF-I or LR^3IGF-I treatment on the expression of IGF-I and IGFBP-3 mRNA in different tissues.
- if combining pGH treatment with IGF-I or LR^3IGF-I has a synergistic effect on growth performance in pigs.

8.2 The effects of IGF-I or LR^{3}IGF-I infusion on growth performance and components of the GH/IGF-I axis.

Subcutaneous administration of IGF-I to normal rats promotes a dose-dependent increase in body weight (Tomas *et al.*, 1993) whereas IGF-I treatment in pigs has no effect on ADG or feed : gain (chapters 3 and 7) These findings in pigs are also supported in sheep where IGF-I treatment also does not produce an increase in body weight gain (Min *et al.*, 1996). The contrast between rats and pigs is even more pronounced in the response to LR^{3}IGF-I. In the rat, LR^{3}IGF-I increases body weight gain and in the pig reduces ADG (chapter 3). It was thought that the poor growth response observed in the pig was associated with IGF-induced hypoglycaemia. Short term infusion or bolus injection of IGF-I has resulted in hypoglycaemia in humans (Guler *et al.*, 1987) rats (Zapf *et al.*, 1986) and pigs (Walton *et al.*, 1989b). In addition, pigs treated with LR^{3}IGF-I showed significantly reduced feed intake (chapter 3), or died (chapter 7) indicating that a fasting hypoglycaemic effect may have contributed to the reduction in ADG. Analysis of plasma glucose levels indicate that this is not the case, as plasma glucose levels were not significantly affected by treatment with IGF-I or LR^{3}IGF-I for 4 days, even though feed intake was reduced in LR^{3}IGF-I treated pigs (chapter 3). This absence of hypoglycaemia has also been observed in previous studies where plasma glucose levels were not affected by treatment with several different doses of LR^{3}IGF-I, (Walton, unpublished observations). It is possible that the death of the pigs treated with LR^{3}IGF-I for 14 days (chapter 7) may be related to hypoglycaemia induced by prolonged fasting as a result of a loss of appetite observed in these animals especially since other components of the endocrine system, in particular insulin, are suppressed.

I postulated that inhibition of components of the GH/IGF-I axis caused by infusion of IGF-I, and particularly, LR^{3}IGF-I, were responsible for poor growth performance in pigs. IGF-I has been shown to regulate GH secretion from rat pituitary cells *in vitro* (Yamashita and Melmed, 1986) and by intracerebroventricular administration in rats (Abe *et al.* 1983) In pigs, both IGF-I and LR^{3}IGF-I infusion suppress plasma GH levels, with LR^{3}IGF-I also reducing the peak area of the GH pulses (chapter 3). Plasma GH concentrations are also suppressed during IGF-I infusion in man (Bermann *et al.*, 1994) and sheep (Fletcher *et al.*, 1995). It is not known whether IGF infusion has the same effect on plasma GH concentrations in the rat as it does in the pig. To date there has been no study on the effect of systemic IGF-I or LR^{3}IGF-I infusion on pulsatile GH expression in rats. This makes comparison between

effects of IGF infusion on pulsatile GH expression and the different growth responses between the two species difficult.

In order to determine if reduced plasma GH levels were a result of GH secretion from the pituitary, IGF-I and LR^{3}IGF-I treated pigs were challenged with an injection of GRF and plasma GH profiles were analysed for 60 minutes following the injection. In a separate study, pituitary GH content was also analysed. GH release from the pituitary was analysed by measuring the size of the plasma GH pulse in response to a GRF challenge and pituitary GH content was analysed by measuring GH content in homogenised whole pituitaries. Neither IGF-I or LR^{3}IGF-I treatment had a significant effect on GH release from the pituitary following the GRF challenge (chapter 3), and pituitary GH content was also not significantly different for IGF-I treated pigs (chapter 7). This would indicate that IGF-I and LR^{3}IGF-I do not act by inhibiting GH secretion but reduce plasma GH levels by suppressing GH mRNA expression or stability. Analysis of pituitary GH mRNA levels in IGF-I and LR^{3}IGF-I treated pigs would shed further light on this.

Treatment with LR^{3}IGF-I reduced plasma IGF-I and IGFBP-3 concentrations (chapters 3, 6 and 7). This reduction in plasma IGF-I is not seen in rats where plasma IGF-I levels remain within the normal range (Tomas *et al.* 1993), and plasma IGFBP-3 levels are elevated with LR^{3}IGF-I treatment (Tomas *et al.*, 1992b). These different responses in rats and pigs may in part explain the poor growth response seen in pigs.

Reduced plasma insulin levels are commonly associated with IGF-I treatment, and result in increased lipid oxidation by releasing free fatty acids from adipose tissue. In the present study, plasma insulin concentrations were decreased with IGF-I and LR^{3}IGF-I treatment, suggesting that IGF peptides, by decreasing GH levels, also modulate the effects of GH on insulin action and glucose metabolism.

8.3 Regulation of IGF-I and IGFBP-3 mRNA by GH in different porcine tissues

The ability for pGH treatment to enhance growth performance in pigs has been well documented (Etherton *et al*, 1986; Campbell *et al*, 1988, Klindt *et al*, 1992). It is widely accepted that the growth promoting actions of GH are mediated via IGF-I, and is therefore not surprising that plasma IGF-I and IGFBP-3 levels are elevated with GH treatment (Glasscock *et al.*, 1991). A number of studies have shown that this elevation in plasma IGF-I is due to increased expression of IGF-I mRNA in different porcine tissues. In pigs, an energy

induced reduction in growth rate is associated with decreased IGF-I *class 1* and *class 2* mRNA expression in liver (Weller *et al.*, 1994). Recent studies have shown that pGH administration in pigs increases IGF-I *class 1* mRNA expression in liver, semitendinosus muscle and adipose tissue but not in longissimus dorsi muscle (Brameld *et al.,* 1996). In the present study, treatment with pGH at a dose of 70 µg/kg/day increased both IGF-I *class 1* and IGF-I *class 2* mRNA in .porcine liver but did not affect IGF-I mRNA expression in kidney, longissimus dorsi, stomach or small intestine (chapter 5).

IGF-I *class 2* transcripts are more sensitive to changes in GH status than IGF-I *class 1* transcripts. This is evident by the positive correlation with liver IGF-I *class 2* mRNA and plasma IGF-I levels in pigs whose growth rate has been manipulated (Weller *et al.*, 1994) and the lack of response of IGF-I *class 1* mRNA levels to moderate doses (30 or 50 µg/kg/day) of pGH (chapter 7; Brameld *et al.*, 1996) compared to increased levels of IGF-I *class 1* mRNA expression at higher doses (70 µg/kg/day) of pGH (chapter 5). In the present study, pGH treatment at a dose of 70 µg/kg/day resulted in a four fold increase in liver IGF-I *class 1* mRNA expression, while IGF-I *class 2* transcripts were increased eight fold. This supports previous findings that IGF-I *class 2* transcripts are the primary source of endocrine regulated IGF-I. The virtual absence of IGF-I *class 2* transcripts in muscle (Weller *et al.*, 1994) and the absence of any detectable amount of IGF-I *class 2* transcripts in kidney (chapter 5, data not shown) suggests that the liver is the major source of endocrine regulated IGF-I.

The increased expression of IGF-I mRNA may be enhanced by an increase in hepatic GH receptors. The levels of GH receptor mRNA have been shown to be positively correlated with growth rate (Weller *et al.*, 1994) and nutritional status (Dauncey *et al.*, 1994) while GH treatment has been shown to increase $[^{125}I]$-bGH binding to hepatic membranes (Ambler *et al.*, 1992). In liver and semitendinosus muscle, IGF-I and GH receptor gene expression have been shown to be positively correlated in pGH treated pigs (Brameld *et al.,* 1996).

Regulation of IGFBP-3 mRNA expression by GH has not been previously examined in the pig. IGFBP-3 mRNA has been detected in porcine gonadal tissue, brain, liver, muscle, lung and kidney (Shimasaki *et al.*, 1990; Lee *et al.*, 1993). GH treatment increases plasma IGFBP-3 levels in rats (Clemmons *et al.*, 1989), humans (Laron, 1993) and pigs (Walton and Etherton, 1989). In the present study in the pig, IGFBP-3 gene expression was most abundant in liver and kidney and increased with pGH treatment in both tissues (chapter 5). Exogenous administration of IGF-I in pigs did not change the levels of IGFBP-3 mRNA in liver or kidney (chapters 6 & 7). In addition, moderate doses of pGH (30 µg/kg/day) also did not affect IGFBP-3 mRNA levels in these organs (chapter 7). This suggests that GH is the major

regulator of IGFBP-3 mRNA expression, although others have shown that IGF-I can stimulate IGFBP-3 in the absence of GH (Camacho-Hubner *et al.*, 1991).

8.4 Effects of IGF-I or LR^{3}IGF-I treatment on the expression of IGF-I and IGFBP-3 mRNA in different tissues.

I have shown that IGF-I and LR^{3}IGF-I treatment in pigs decreased plasma GH levels and treatment with LR^{3}IGF-I also decreased plasma IGF-I and IGFBP-3 levels (chapter 3). To determine if the decrease in plasma protein levels was due to decreased IGF-I and/or IGFBP-3 gene expression, IGF-I and IGFBP-3 mRNA expression was investigated in the organs that had been shown to express IGF-I and/or IGFBP-3 in a GH dependent manner (chapter 5). In liver, neither IGF-I nor LR^{3}IGF-I administered for four days had a significant effect on IGF-I *class 1* mRNA expression (chapter 6), although increasing the treatment period to two weeks resulted in a significant decrease in liver IGF-I *class 1* mRNA expression (chapter 7). The effect of LR^{3}IGF-I treatment for two weeks could not be examined due to pig mortality. This time dependent response in IGF-I *class 1* mRNA expression may be due to the decreased sensitivity of expression of this transcript to GH status. Liver IGF-I *class 2* mRNA expression was significantly reduced in IGF-I and LR^{3}IGF-I treated pigs (chapter 6). This decrease in liver IGF-I *class 2* mRNA was also observed in pigs that had been treated with IGF-I for 14 days. It is evident that LR^{3}IGF-I treated pigs show a reduction in plasma IGF-I levels due to decreased IGF-I mRNA expression. IGF-I treated pigs have increased levels of plasma IGF-I, which is entirely due to the exogenously administered growth factor, since IGF-I mRNA levels are suppressed in IGF-I treated pigs.

Treatment with pGH increased IGFBP-3 mRNA expression in porcine liver and kidney (chapter 5), indicating that IGFBP-3 mRNA expression is GH-dependent in these tissues. Plasma IGFBP-3 levels are not affected by IGF-I treatment and are reduced with LR^{3}IGF-I treatment (chapters 3, 6 & 7). This decrease in plasma IGFBP-3 levels is not due to decreased gene expression, since neither IGF-I nor LR^{3}IGF-I treatment affect IGFBP-3 mRNA levels in porcine liver or kidney (chapter 6 & 7). The most likely cause for decreased plasma IGFBP-3 levels in LR^{3}IGF-I treated pigs is post-translational modification of the binding protein by IGFBP-3 proteases. The levels of protease activity in these plasma samples was not examined but would be expected to be increased in LR^{3}IGF-I treated pigs.

8.5 Synergistic effects of combination pGH treatment with IGF-I or LR^{3}IGF-I

A combination of GH and IGF-I treatment has shown synergistic actions on body weight gain in uraemic rats (Hazel *et al.*, 1994), calorie restricted healthy male volunteers (Kupfer *et al.*, 1993) and hypophysectomised rats (Fielder *et al.*, 1996). In the pig, the combination treatment of IGF-I and pGH did not have added beneficial effects on growth performance in general (chapters 3 & 7) with the exception of one study, where pigs receiving the combined treatment showed a transient interactive effect on ADG after one week of treatment. Plasma hormone levels or their mRNA expression where also not improved when compared to GH treatment alone. This is most evident when comparing the effects of IGF-I, pGH and combined treatment on the expression of both IGF-I transcripts. Liver IGF-I *class 1* and IGF-I *class 2* mRNA levels are suppressed with IGF-I treatment (chapter 7) and evidence has been presented in this thesis that would suggest that IGF-I *class 2* mRNA expression is more sensitive to pGH treatment than IGF-I *class 1* mRNA expression. It is then not surprising that pGH treatment at a moderate dose for two weeks does not affect IGF-I *class 1* mRNA but significantly increases IGF-I *class 2* mRNA (chapter 7). Since IGF-I *class 1* expression is not affected by pGH treatment alone, there is no compensatory effect on the decreased IGF-I *class 1* mRNA levels with IGF-I treatment when both peptides are administered together. In contrast, the decrease in liver IGF-I *class 2* mRNA expression seen with IGF-I treatment is compensated (but not improved above that of pGH treatment alone) by co-administration with pGH (chapter 7).

The effect of combination treatment on plasma IGFBP-3 levels is somewhat different. Neither IGF-I or pGH administered for 4 days altered plasma IGFBP-3 levels, but when administered together, IGFBP-3 levels were significantly elevated (chapter 3). In a separate study, where pigs were treated for 2 weeks, pGH significantly increased plasma IGFBP-3 levels when administered alone and in combination with IGF-I. Thus it appears that synergistic actions of combination treatments are dependent on length of administration. LR^{3}IGF-I treatment alone and in combination with pGH showed a different response on plasma IGFBP-3 levels. Plasma IGFBP-3 levels were significantly reduced with LR^{3}IGF-I treatment and remained suppressed with the combination treatment (chapter 3). Plasma insulin and plasma GH levels also did not benefit from possible synergistic actions of pGH treatment in combination with IGF-I or LR^{3}IGF-I since they remained suppressed when IGF peptides were administered alone or in combination with pGH. It appears that combination treatment may be a successful treatment regime in catabolic but not normal states, since it has been shown to

have synergistic actions on growth performance in uraemic rats (Hazel *et al.*, 1994), calorie restricted volunteers (Kupfer *et al.*, 1993) and hypophysectomised rats (Fielder *et al.*, 1996) but the effects on normal pigs is less clear.

In conclusion, why rats and pigs have such different growth responses to treatment with IGF-I or LR^3IGF-I is not entirely clear although these experiments provide further evidence to resolve the issue. In order to increase growth performance in the rat, doses of 2 mg/kg/day are commonly used (Hazel *et al.*, 1994), whereas in pigs growth performance is improved with doses as low as 50 µg/kg/day (Chung *et al.*, 1985). This may suggest that the growth promoting actions of IGF-I are not very sensitive to GH status in rats. If IGF peptides do reduce plasma levels of GH in the rat as they do in the pig, the growth promoting actions of IGF-I may not be impaired in rats. This is evident from the fact that treatment with LR^3IGF-I does not affect plasma IGF-I and increases plasma IGFBP-3 levels in the rat (Tomas *et al.*, 1992 & 1993), but decreases plasma IGF-I and IGFBP-3 levels in the pig (chapter 3 & 6). Clearly, the increased potency of LR^3IGF-I in rats and pigs is related to the poor binding capacity of this peptide for IGFBP-3.

Free IGF-I has been shown to cross the blood brain barrier (Reinhardt and Bondy, 1994). LR^3IGF-I, which does not readily bind IGFBP-3 may therefor be able to rapidly cross the blood brain barrier to affect GH secretion or expression via the hypothalamus as well as at the level of the pituitary. It may also have an effect on the central brain stem to reduce appetite in pigs. IGF-I, being mostly bound with IGFBP-3 in a 150 kDa complex is not able to cross the blood brain barrier and may therefore only be able to act at the level of the pituitary to regulate GH secretion and/or synthesis.

8.6 Future

In order to verify the trends observed especially those for the expression of IGF-I *class 1* mRNA in porcine liver and kidney for short term treatment of IGF-I and LR^3IGF-I (chapter 6), similar experiments need to be performed with an increased number of animals (n = 6) per treatment group. Due to the nature of the experiments this was not possible at the time, but would clearly have benefited the outcome of the experiments by decreasing the standard error within a treatment group.

Results presented in chapter 3 show that IGF-I and LR^3IGF-I treatment decrease plasma GH levels in pigs. Whether this is achieved by reducing expression of GH mRNA in the pituitary or inhibiting GH secretion is not clear. It is also not known whether the IGF

effects are primarily at the level of the pituitary or hypothalamus. To address the first issue, GH mRNA expression in porcine pituitaries can be easily measured using similar methods to those described in this thesis. Evidence in the literature exists that indicates IGF-I affects somatostatin and GRF expression *in vitro* (Sato and Frohman, 1993; Uchiyama *et al.*, 1994). Whether a similar situation exists *in vivo* may be determined by measuring the expression of somatostatin and GRF mRNA in hypothalamic tissue from pigs treated with IGF peptides. *In vitro* culture systems may also been used whereby hypothalamic slices are incubated in chambers connected to separate culture dishes containing pituitary cells. In such situations hypothalamic/pituitary effects of IGF peptide treatment can be studied *in vitro*. Setting up such a system is by no means a small task and was outside the time frame of the present study.

There are a number of studies in the literature that indicate a close correlation exists between plasma IGF-I and hepatic IGF-I mRNA levels (Maiter *et al.*, 1992; Bichell *et al.*, 1992; Jewel *et al.*, 1989; Mesiano *et al.*, 1989). Whether the abundance of GH receptors play an important role in the regulation of hepatic IGF-I expression in IGF peptide treated pigs is not known. The presence of GH receptors can be assessed by estimating the extent of GH binding to liver membrane preparations as well as measuring the changes in GH receptor mRNA expression in IGF peptide treated pigs.

Finally, it would be interesting to address the issue of the differences in GH sensitivity in response to IGF peptides between rats and pigs. If indeed the differences in growth response are due to the relative insensitivity of GH expression to IGF-I levels in the rat, then it would be expected that plasma GH levels and the magnitude of GH pulses would not be decreased by LR^3IGF treatment in rats as they are in pigs. To determine if this is the case, a experiment similar to that described in chapter 3 would also need to be performed in rats.

CHAPTER 9

REFERENCES

Abe H, Molitch M.E., Van Wyk J.J. and Underwood L.E. (1983) Human growth hormone and somatomedin C suppress the spontaneous release of growth hormone in unanesthetized rats. *Endocrinology* 113 1319-1324

Adamo M.L., Ben-Hur H., Roberts Jr C.T. and LeRoith D. (1991) Regulation of start site usage in the leader exons of the rat insulin-like growth factor-I gene by development, fasting and diabetes. *Molecular Endocrinology* **124**, 2737-2744

Adamo M.L., Koval A., LeRoith D. and Roberts C.T. (1994) Posttranscriptional regulation of IGF-I gene expression. In *The insulin-like growth factors and their regulatory proteins.* pp 23-31 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld R.G. Elsevier Science Publishing Co. Inc., Amsterdam

Adamo M.L., Lowe W.L. Jr., LeRoith D. and Roberts C.T. Jr. (1989) Insulin-like growth factor I messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development of the rat. *Endocrinology* **124**, 2737-2744

Adashi E.Y., Resnick C.E., D'Ercole A.J., Svoboda M.E. and Van Wyk J.J. (1985) Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocrine Reviews* **6**, 400-420

Aguila M.C. (1994) Growth hormone-releasing factor increases somatostatin release and mRNA levels in the rat periventricular nucleus via nitric oxide by activation of guanylate cyclase. *Proceedings of the National Academy of Sciences*, USA **91**, 782-786

Aguila M.C., Boggaram V. and McCann S.M. (1993) Insulin-like growth factor I modulates hypothalamic somatostatin through a growth hormone releasing factor increased somatostatin release and messenger ribonucleic acid levels. *Brain Research* **625**, 213-218

Aguila M.C. and McCann S.M. (1988) Calmodulin dependence of somatostatin release stimulated by growth hormone-releasing factor. *Endocrinology* **123**, 305-309

Albiston A.L. and Herington A.C. (1992) Tissue distribution and regulation of insulin-like growth factor (IGF)-binding protein-3 messenger ribonucleic acid (mRNA) in the rat: Comparison with IGF-I mRNA expression. *Endocrinology* **130**, 497-502

Ambler G.R., Breier B.H., Surus A., Blair H.T., McCutcheon S.N., Silbergeld A. and Gluckman P.D. (1992) The interrelationship between and the regulation of hepatic growth hormone receptors and circulating GH binding protein in the pig. *Acta Endocrinologica* **126** 155-161

Andres C.J., Green M.L., Clapper J.A., Cline T.R., Diekman M.A. and Thomford P.J. (1993) Influence of porcine somatotropin on endocrine and histological variables in gilts. *Journal of Animal Science* **71**, 1552-1560

Andress D.L. and Birnbaum R.S. (1991) A novel human insulin-like growth factor binding protein secreted by osteoblast cells. *Biochemical and Biophysical Research Communications* **176**, 213-218

Argente J., Barrios V., Pozo J., Munoz M.T., Hervas F., Stene M. and Hernandez M. (1993) Normative data for insulin-like growth factors (IGFs), IGF-binding proteins, and growth hormone binding protein in a healthy spanish pediatric population: age and sex-related changes. *Journal of Clinical Endocrinology and Metabolism* **77**, 1522-1528

Armstrong J.D., Cohick W.S., Harvey R.W., Heimer E.P. and Campbell R.M. (1993) Effect of feed restriction on serum somatotropin, insulin-like growth factor-I and IGF binding proteins in cyclic heifers actively immunized against growth hormone releasing factor. *Domestic Animal Endocrinology* **10** 315-324

Armstrong J.D., Esbenshade K.L., Johnson J.L., Coffey M.T., Heimer E., Campbell R.M., Mowles T. and Felix A. (1990) Active immunization of pigs against growth hormone-releasing factor: Effect on concentrations of growth hormone and insulin-like growth factor 1. *Journal of Animal Science* **68**, 427-434

Arner P., Sjoberg S., Gjotterberg M. and Skottner A. (1989) Circulating insulin-like growth factor I in type 1 (insulin-dependent) diabetic patients with retinopathy. *Diabetologia* **32**, 753-758

Ashton I.K., Zapf J., Einschenk I. and MacKenzie I.Z. (1985) Insulin-like growth factor (IGF) I and II in human foetal plasma and relationship to gestational age and foetal size during mid-pregnancy. *Acta Endocrinologica* **110**, 558-563

Atkinson K., Hill D.J., Strain A.J. and Miner R.D.G. (1987) Evidence that the mechanism of action of insulin-like growth factors (IGF) differs between fetal and postnatal life. *Journal of Endocrinology* **112** (Suppl.), Abstr. 62

Bach L.A., Thotakura N.R. and Rechler M.M. (1993) Human insulin-like growth factor-6 is O-glycosylated. *Growth Regulation* **3**, 59-62

Bach M.A. and Bondy C.A. (1993) The insulin-like growth factor system in the human pituitary. *Endocrine Journal* **1**, 187-191

Baker J., Liu J., Robertson E.J. and Efstratiadis A. (1993) Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75**, 73-82

Bale L.K. and Conover C.A. (1992) Regulation of insulin-like growth factor binding protein-3 messenger ribonucleic acid expression by insulin-like growth factor I. *Endocrinology* **131**, 608-614

Ballard F.J., Knowles S.E., Walton P.E., Edson K., Owens P.C., Mohler M.A. and Ferraiolo B.L. (1991) Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I), IGF-II and des (1-3) IGF-I in rats. *Journal of Endocrinology* **128**, 197-204

Bang P. and Hall K. (1992) Insulin-like growth factors as endocrine and paracrine hormones. In The insulin-like growth factors: Structure and biological functions. Schofield P.N. Editor pp151-177

Bang P., Brismar K. and Rosenfeld R.G. (1994) Increased proteolysis of insulin-like growth factor binding protein 3 (IGFBP-3) in non-insulin dependent diabetes mellitus serum with elevation of a 29 kilo-dalton (kDa) glycosylated IGFBP-3 fragment contained in the approximately 130- to 150- kDa ternary complex. *Journal of Clinical Endocrinology and Metabolism* **78**, 1119-1127

Bar R.S., Boes M., Clemmons D.R., Busby W.H., Sandra A., Dake B.L. and Booth B.A. (1990) Insulin differentially alters transcapillary movement of intravascular IGFBP-1, IGFBP-2 and endothelial cell IGF-binding protein in the rat heart. *Endocrinology* **127**, 497-509

Barinaga M., Bilezikjian L.M., Vale W.W., Rosenfeld M.G. and Evans R.M. (1985) Independent effects of growth hormone releasing factor on growth hormone release and gene transcription. *Nature* **314**, 279-281

Barreca A. and Minuto F. (1989) Somatomedins: chemical and functional characteristics of the different molecular forms. *Journal of Endocrine Investigations* **12**, 279-293

Bass J.J., Oldman J.M., Hodgkinson S.C., Fowke P.J., Sauerwein H., Molan P., Breier B.H. and Gluckman P.D. (1991) Influences of nutrition and bovine growth hormone (GH) on hepatic GH binding, insulin-like growth factor-I and growth of lambs. *Journal of Endocrinology* **128** 181-186

Bastian S.E.P., Walton P.E., Wallace J.C. and Ballard F.J. (1993) Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I) and an analogue LR^3IGF-I in pregnant rats. *Journal of Endocrinology* **138**, 327-336

Baumann G. (1991) Growth hormone heterogeneity: Genes, isohormones, variants, and binding proteins. *Endocrine Review* **12**, 424-449

Baxter R.C. (1988) The insulin-like growth factors and their binding proteins. *Comparative Biochemistry and Physiology* **91**, 229-235

Baxter R.C. and Cowell C.T. (1987) Diurnal rhythm of growth hormone-independent binding protein for insulin-like growth factors in human plasma. *Journal of Clinical Endocrinology and Metabolism* **65**, 432-440

Baxter R.C., Hizuka N., Takano K., Holman S.R. and Asakawa K. (1993) Responses of insulin-like growth factor binding protein-1 (IGFBP-1) and the IGFBP-3 complex to administration of insulin-like growth factor-I. *Acta Endocrinologica* **128**, 101-108

Baxter R.C. and Martin J.L. (1986) Radioimmunoassay of growth hormone dependent insulin-like growth hormone-dependent insulin-like growth factor binding protein in human plasma. *Journal of Clinical Investigation* **78**,1504-1512

Baxter R.C. and Martin J.L. (1987) Binding proteins for insulin-like growth factors in adult rat serum: comparison with other human and rat binding proteins. *Biochemical and Biophysical Research Communications* **147**, 408-415

Baxter R.C. and Martin J.L. (1989) Structure of the M_r 140 000 growth hormone dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labelling. *Proceedings of the National Academy of Sciences, USA* **86**, 6898-6902

Baxter R.C., Martin J.L. and Beniac V.A. (1989) High molecular weight insulin-like growth factor binding protein complex: purification and properties of the acid-labile subunit from human serum. *Journal of Biological Chemistry* **264**, 11843-11848

Baxter R.C., Martin J.L. and Wood M.H. (1987) Two immunoreactive binding proteins for insulin-like growth factors in human amniotic fluid: relationship to fetal maturity. *Journal of Clinical Endocrinology and Metabolism* **65**, 423-431

Bayne M.L., Applebaum J., Underwood D., Chicchi G.G., Green B.G., Hayes N.S. and Cascieri M.A. (1989)The C region of human insulin-like growth factor (IGF) -I is required for high affinity binding to the type 1 IGF receptor. *The Journal of Biological Chemistry* **264**, 11004-11008

Beck F., Samani N.J., Penschow J.D., Thorley B., Tregaer G.W. and Coghan J.P. (1987) Histochemical localization of IGF-I and -II mRNA in the developing rat embryo. *Development* **101**, 175-184

Bell G.I., Merryweather J.P., Sanchez-Pescador R., Stempien M.M., Priestly L., Scott J. and Rall L.B. (1984) Sequence of a cDNA clone encoding human preproinsulin-like growth factor II *Nature* **310**, 775-777

Bell G.I., Stempien M.M., Fong N.M. and Rall L.B. (1986) Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. *Nucleic Acids Research* **14,** 7873-7882

Bell G.I., Stempien M.M., Fong N.M. and Seino S. (1990) Sequence of a cDNA encoding guinea pig IGF-I. *Nucleic Acids Research* **18,** 4275

Berekt A., Lang C.H., Fan J., Frost R.A. and Wilson T.A. (1995) Insulin-like growth factor binding protein (IGFBP)-3 proteolysis in children with insulin-dependent diabetes

mellitus: a possible role for insulin in the regulation of IGFBP-3 protease activity. *Journal of Clinical Endocrinology and Metabolism* **80**, 2282-2288

Bermann M., Jaffe C.A., Tsai W., DeMott-Friberg R. and Barkan A. (1994) Negative feedback regulation of pulsatile growth hormone secretion by insulin-like growth factor I. Involvement of hypothalamic somatostatin. *The Journal of Clinical Investigation* **94** 138-145.

Bhaumic, B., Bala R.M. and Hollenberg M.D. (1981) Somatomedin receptor of human placenta: solubilization, photolabeling, partial purification and comparison with insulin receptor. *Proceedings of the National Academy of Sciences, USA* **78**, 4279-4283

Bianda T.L., Hussain M.A., Keller A., Glatz Y., Schmitz O., Christiansen J.S., Alberti K.G.M.M. and Froesch E.R. (1996) Insulin-like growth factor-I in man enhances lipid mobilization and oxidation induced by growth hormone pulse. *Diabetologia* **39**, 961-969

Bichell D.P., Kikuchi K. and Rotwein P. (1992) Growth hormone rapidly activates insulin-like growth factor I gene transcription *in vivo*. *Molecular Endocrinology* **6**, 1899-1908

Binoux M., Lalou C., Lassarre C. and Segovia B. (1994) Regulation of IGF bioavailability by IGFBP proteases. In *The insulin-like growth factors and their regulatory proteins*. pp 217-226 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld R.G. Elsevier Science Publishing Co. Inc., Amsterdam

Blum W.F., Albertsson-Wikland K., Rosberg S. and Ranke M.B. (1993a) Serum levels of insulin-like growth factor I (IGF-I) and IGF binding protein 3 reflect spontaneous growth hormone secretion. *Journal of Clinical Endocrinology and Metabolism* **76**, 1610-1616

Blum W.F., Horn N., Kratzsch J., Jørgensen J.O.L., Juul A., Teale D., Mohnike K. and Ranke M.B. (1993b) Clinical studies of IGFBP-2 by radioimmunoassay. *Growth Regulation* **3**, 100-104

Blum W.F., Jenne E.W., Reppin F., Kietzmann K., Ranke M.B. and Bierich J.R. (1989) Insulin-like growth factor I (IGF-I) binding protein complex is a better mitogen than free IGF-I. *Endocrinology* **125**, 766-772

Blum W.F., Ranke M.B., Kietzman K., Gauggel E., Zeisel H.J. and Bierich J.R. (1990) A specific radioimmunoassay for the growth hormone (GH)-dependent somatomedin-binding protein: its use for diagnosis of GH deficiency. *Endocrinology* **70**, 1292-1298

Bohannon N., Figlewicz D., Corp E., Wilcox B., Porte Jr., D. and Baskin D. (1986) Indentification of binding sites for an insulin-like growth factor (IGF-I) in the median eminence of the rat brain by quantitative autoradiography. *Endocrinology* **119**, 943-945

Boisclair Y.R., Dunshea F.R., Bell A.W., Bauman D.E. and Harkins M. (1989) Effect of bovine somatotropin on glucose metabolism in steers. *FASEB Journal* **3**, abstract 938

Borsi L., Rosenfeld R.G., Liu F. and Hintz R.L. (1982) Somatomedin peptide distribution and somatomedin-binding protein content in cord plasma: comparison to normal and hypopituitary plasma. *Journal of Clinical Endocrinology and Metabolism* **54**, 223-228

Boulle N., Schneid H., Listrat A., Holthuizen P., Binoux M. and Groyer A. (1993) Developmental regulation of bovine insulin-like growth factor-II (IGF-II) gene expression: homology between bovine transcripts and human IGF-II exons. *Journal of Molecular Endocrinology* **11**, 117-128

Brameld J.M., Atkinson J.L., Saunders J.C., Pell J.M., Buttery P.J. and Stewart R.G. (1996) Effects of growth hormone administration and dietary protein intake on insulin-like growth factor I and growth hormone receptor mRNA expression in porcine liver, skeletal muscle, and adipose tissue. *Journal of Animal Science* **74**, 1832-1841

Braulke T., Tippmer S., Chao H-J. and von Figura K.(1990) Regulation of the mannose 6-phosphate/insulin-like growth factor II receptor distribution by activators and inhibitors of protein kinase C. *European Journal of Biochemistry* **189**, 609-616

Breier B.H., Gluckman P.D. and Bass J.J. (1985) The somatotrophic axis in young steers: influence of nutritional status and oestadiol-17β on hepatic high- and low-affinity somatotrophic binding sites. *Journal of Endocrinology* **116**, 169-177

Breier B.H., Gluckman P.D. and Bass J.J. (1988) Influence of nutritional status and oestradiol-17 beta on plasma growth hormone, insulin-like growth factors-I and -II and the response to exogenous growth hormone in young steers. *Journal of Endocrinology* **118**, 243-250

Brice A.L., Cheetham J.E., Bolton V.N., Hill N.C.W. and Schofield P.N. (1989) Temporal changes in the expression of the insulin-like growth factor II gene associated with tissue maturation in the human fetus. *Development* **106**, 543-554

Brinkman A., Groffen C., Kortleve D.J. Guerts van Kessel A. and Drop S.L. (1988) Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IGFBP-1). *EMBO Journal* **7**, 2417-2423

Brismar K., Grill V., Efendic S. and Hall K. (1991) The insulin-like growth factor binding protein-1 in low and high insulin responders before and during dexamethasone treatment. *Metabolism* **40**, 728-732

Brismar K. and Hall K (1993) Clinical applications of IGFBP-1 and its regulation. *Growth Regulation* **3**, 98-100

Brisenden J.E., Ullrich A. and Francke U. (1984) Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. *Nature* **310**, 781-784

Bryan K.A., Hagen D.R. and Hammond J.M. (1992) Effect of frequency of administration of exogenous porcine growth hormone on growth and carcass traits and ovarian function of prepubertal gilts. *Journal of Animal Science* **70**, 1454-1463

Bryan K.A., Hammond J.M., Canning S., Mondschein J., Carbaugh D.E., Clark A.M. and Hagen D.R. (1989) Reproductive and growth responses of gilts to exogenous porcine pituitary growth hormone. *Journal of Animal Science* **67**, 196-205

Buonomo F.C. and Baile C.A. (1991) Influence of nutritional deprivation on insulin-like growth factor I, somatotropin, and metabolic hormones in swine. *Journal of Animal Science* **69** 755-760

Burrin D.G., Wester T.J., Davis T.A., Amick S. and Heath J.P. (1996) Orally administered IGF-I increases intestinal mucosal growth in formula-fed neonatal pigs. *Journal of American Physiology* **270**, R1085-R1091

Calabresi E., Ishikawa E., Bartolini L., Delitala G., Fanciulli G., Oliva O., Veldhuis J.D. and Serio M. (1996) Somatostatin infusion suppresses GH secretory burst frequency and mass in normal men. *American Journal of Physiology Endocrinology and Metabolism* **33**, E975-E979

Camacho-Hubner C., Clemmons D.R. and D'Ercole A.J. (1991) Regulation of insulin-like growth factor (IGF) binding proteins in transgenic mice with altered expression of growth hormone and IGF-I. *Endocrinology* **129**, 1201-1206

Campbell, R.G., Steele N.C., Caperna T.J., McMurtry J.P., Solomon M.B., and Mitchell A.D.(1988). Interrelationships between energy intake and endogenous porcine growth hormone administration on the performance, body composition and protein and energy metabolism of growing pigs weighing 25 to 55 kilograms live weight. *Journal of Animal Science* **66** 1643

Campbell R.G., Steele N.C., Caperna T.J., McMurtry J.P., Solomon M.B. and Mitchell A.D. (1989) Interrelationships between sex and exogenous growth hormone administration on performance, body composition and protein and fat accretion of growing pigs. *Journal of Animal Science* **67**, 177-186

Carlsson L.M.S., Clark R.G. and Robinson I.C.A.F. (1990) Sex differences in growth hormone feedback in the rat. *Journal of Endocrinology* **126**, 27-35

Carlsson-Skwirut C., Jornvall H., Holmgren A., Andersson C., Bergman J., Lundquist G., Sjogren B. and Sara V.R. (1986) Isolation and characterization of variant IGF-1 as well as IGF-2 from adult human brain. *FEBS Letters* **201**, 46-50

Caro J.F., Poulos J., Ittoop O., Pories W.J., Flickinger E.G. and Sinha M.K. (1988) Insulin-like growth factor I binding in hepatocytes from human liver, human hepatoma, and normal, regenerating, and fetal rat liver. *Journal of Clinical Investigation* **81**, 976-981

Carson M.J., Behringer R.R., Brinster R.L. and McMorris F.A. (1993) Insulin-like growth factor-I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* **10**, 729-740

Cascieri M.A., Chicchi G.G., Applebaum J., Green B.G., Hayes N.S. and Bayne M.L. (1989) Structural analogs of human insulin-like growth factor (IGF) I with altered affinity for type-2 IGF receptors. *Journal of Biological Chemistry* **264**, 2199-2202

Cascieri M.A., Chicchi G.G., Applebaum J. Hepes N.S., Given B.G. and Bayne M.L. (1988) Mutants of human insulin-like growth factor-I with reduced affinity for the type 1 insulin-like growth factor receptor. *Biochemistry* **27**, 3229-3233

Chan W., Krieg R.J., Sayles T.E. and Matt D.W. (1993) Caloric restriction and expression of liver insulin-like growth factor-I, growth hormone receptor and pituitary growth hormone. *Nutrition Research* **13**, 1343-1350

Chard T. (1989) Hormonal control of growth in the human fetus. *Journal of Endocrinology* **123**, 3-9

Chernausek S.D., Jacobs, S. and Van Wyk J.J. (1981) Structural similarities between human receptors for somatomedin C and insulin: Analysis by affinity labelling. *Biochemistry* **20**, 7345-7350

Chihara K., Minamitani N., Kaji H., Arimura A. and Fujita T. (1981) Intraventricular injected growth hormone stimulates somatostatin release into rat hypophyseal portal blood. *Endocrinology* **109**, 2279-2281

Chow J.C., Murray J.D., Pomp D., Baldwin R.L., Calvert C.C. and Oberbaum A.M. (1994) Regulation of insulin-like growth factor-I and binding protein-3 expression in oMtla-oGH transgenic mice. *Transgenic Research* **3**, 127-133

Christiansen J.S., Gammelgaard J., Orskov H. Andersen A.R., Telmer S. and Parving H.H. (1981) Kidney function and size in normal subjects before and during growth hormone administration for one week. *European Journal of Clinical Investigation* **11** 487-490

Chung C.S., Etherton T.D. and Wiggins J.P. (1985) Stimulation of swine growth by porcine growth hormone. *Journal of Animal Science* **60**, 118-130

Clairmont K.B. and Czech M.P. (1989) Chicken and *Xenopus* mannose 6-phosphate receptors fail to bind insulin-like growth factor II. *Journal of Biological Chemistry* **264**, 16390-16392

Clarke I.J., Fletcher T.P., Pomares C.C., Holmes J.H.G., Dunshea F., Thomas G.B., Tilbrook A.J., Walton P.E. and Galloway (1993) Effect of high-protein feed supplements on concentrations of growth hormone (GH), insulin-like growth factor-I (IGF-I) and IGF binding protein-3 in plasma and on the amounts of GH and messenger RNA for GH in the pituitary glands of adult rams. *Journal of Endocrinology* **138** 421-427

Clemmons D.R. (1984) Multiple hormones stimulate the production of somatomedin by cultured human fibroblasts. *Journal of Clinical Endocrinology and Metabolism* **58**, 850-856

Clemmons D.R. (1993) IGF binding proteins and their functions. *Molecular Reproduction and Development* **35**, 368-375

Clemmons D.R. (1994) Role of post translational modifications in modifying the biological activity of insulin like growth factor binding proteins. In *Current directions in insulin-like growth factor research.* pp 245-253 Eds. D. LeRoith and M.K. Raizada

Clemmons D.R., Cascieri M.A., Camacho-Hubner C., McCusker R.H. and Bayne M.L. (1990) Discrete alterations of the IGF-I molecule that alter its affinity for IGF binding proteins result in changes in bioavailability. *The Journal of Biological Chemistry* **265**, 12210-12216

Clemmons D.R., Dehoff M.L., Busby W.H., Bayne M.L. and Cascieri M.A. (1992) Competition for binding to insulin-like growth factor (IGF) binding protein-2, 3, 4, and 5 by the IGFs and IGF analogs. *Endocrinology* **131**, 890-895

Clemmons D.R., Klibanski A., Underwood L.E. McArthur J.W., Ridgway E.C., Beitins I.Z. and Van Wyk J.J. (1981) Reduction of plasma immunoreactive somatomedin-C during fasting in humans. *Journal of Clinical Endocrinology and Metabolism* **53**, 1247-1250

Clemmons D.R., Snyder D.K. and Busby W.H. (1991) Variables controlling the secretion of insulin-like growth factor binding protein-2 in normal human subjects. *Journal of Clinical Endocrinology and Metabolism* **73**, 727-733

Clemmons D.R., Thissen J.P., Maes M., Ketelslegers J.M. and Underwood L.E. (1989) Insulin-like growth factor-I (IGF-I) infusion into hypophysectomized or protein deprived rats induces specific IGF binding proteins in serum. *Endocrinology* **125**, 2967-2972

Coleman M.E. and Etherton T.D. (1991) Effects of exogenous porcine growth hormone on serum insulin-like growth factor binding proteins in growing pigs. *Journal of Endocrinology* **128**, 175-180

Coleman M.E., Russell L. and Etherton T.D. (1994) Porcine somatotropin (pST) increases IGF-I mRNA abundance in liver and subcutaneous adipose tissue but not in skeletal muscle of growing pigs *Journal of Animal Science* **72** 918-924

Conlon M.A., Tomas F.M., Owens P.C., Wallace J.C., Howarth G.S. and Ballard F.J. (1995) Long R^3 insulin-like growth factor-I (LR^3IGF-I) infusion stimulates organ growth but reduces plasma IGF-I, IGF-II and IGF binding protein concentrations in the guinea pig. *Journal of Endocrinology* **146** 247-253.

Conover C.A., Clarkson J.T., Wissink S., Durham S.K. and Bale L.K. (1994) Biological effects of IGF regulated IGFBP in cultured human fibroblasts. In *The insulin-like growth factors and their regulatory proteins.* pp 175-182 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld R.G. Elsevier Science Publishing Co. Inc., Amsterdam

Conover C.A., Ronk M., Lombana F. and Powell D.R. (1990) Structural and biological characterization of bovine insulin-like growth factor binding protein-3. *Endocrinology* **127**, 2795-2803

Cooke R.M., Harvey T.S. and Campbell I.D. (1991) Solution structure of human IGF-I: a nuclear magnetic resonance and restrained molecular dynamics study. *Biochemistry* **30**, 5484-5491

Corvera S., Whitehead R.E., Mottola C. *et al.* (1986) The insulin-like growth factor II receptor is phosphorylated by a tyrosine kinase in adipocyte plasma membranes. *Journal of Biological Chemistry* **261**,7675-7679

Cottam Y.H., Blair H.T., Gallaher B.W., Purchas R.W., Breier B.H., McCutcheon S.N. and Gluckman P.D. (1992) Body growth, carcass composition, and endocrine changes in lambs chronically treated with recombinantly derived insulin-like growth factor-I. *Endocrinology* **130**, 2924-2930

Cubbage M.L., Suwanichkul A. and Powell D.R. (1990) Insulin-like growth factor binding protein-3. Organization of the human chromosomal gene and demonstration of promoter activity. *The Journal of Biological Chemistry* **265**, 12642-12649

Cwyfan Hughes S.C., Cotterill A.M., Molloy A.R., Cassell T.B., Braude N., Hinds C.J., Wass J.A. and Holly J.M. (1992) The induction of specific proteases for insulin-like growth factor binding proteins (IGFBPs) following major heart surgery. *Journal of Endocrinology* **135**, 135-145

Dahms N.M., Brzycki-Wessell M.A., Ramanujam K.S. and Seetharam B. (1993) Characterization of mannose 6-phosphate receptors (MPRs) from opossum liver: opossum cation-independent MPR binds insulin-like growth factor-II. *Endocrinology* **133**, 440-446

Daughaday W.H. and Reeder C. (1966) Synchronous activation of DNA synthesis in hypophysectomized rat cartilage by growth hormone. *Journal of Laboratory and Clinical Medicine* **68,** 357-368

Daughaday W.H. and Rotwein (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocrine Reviews* **10**, 68-91

Daughaday W.H., Yanow C.E. and Kapadia M. (1986) Insulin-like growth factors I and II in maternal and fetal guinea pig serum. *Endocrinology* **119**, 49-494

Dauncey M.J., Burton K.A., White P., Harrison A.P., Gilmour R.S., Duchamp C. and Cattaneo D. (1994) Nutritional regulation of growth hormone receptor gene expression. *FASEB Journal* **8** 81-88

Dauncy M.J. and Buttle H.L. (1990) Differences in growth hormone and prolactin secretion associated with environmental temperature and energy intake. *Hormone and Metabolic Research* **22**, 524-527

Davies S.C., Wass J.A.H., Ross R.J. Cotterill A.M., Buchanan C.R., Coulson V.J. and Holly J.M. (1991) The induction of a specific protease for insulin-like growth factor binding protein-3 (IGFBP-3) in the circulation during severe illness. *Journal of Endocrinology* **130**, 469-473

de Bruijne J.J., Eigenmann J.E. and Froesch E.R. (1985) Insulin-like growth factor I and growth hormone in canine starvation. *Acta Endocrinologica* **108** 161

DeChiara T.M., Efstratiadis A. and Robertson E.J. (1990) A growth deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345**, 78-80

Delehaye-Zervas M., Mertani H., Martini J., Nihoul-Feketé C., Morel G. and Postel-Vinay M. (1994) Expression of the growth hormone receptor gene in human digestive tissues. *Journal of Clinical Endocrinology and Metabolism* **78**, 1473-1480

Delhanty P.J.D. and Han V.K.M. (1993) The expression of insulin-like growth factor (IGF) binding protein-2 and IGF-II genes in the tissues of the developing ovine fetus. *Endocrinology* **132**, 41-52

Delhanty P.J.D., Hill D.J., Shimasaki S. and Han V.K.M. (1993) Insulin-like growth factor binding protein-4, -5 and-6 mRNAs in the human fetus: Localization to sites of growth and differentiation? *Growth Regulation* **3**, 8-11

D'Ercole A.J., Stiles A.D. and Underwood L.E. (1984) Tissue concentrations of somatomedin-C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *Proceedings of the National Academy of Sciences, USA* **81,** 935-939

D'Ercole A.J., Wilson D.F. and Underwood L.E. (1980) Changes of the circulating form of somatomedin-C during fetal life. *Journal of Clinical Endocrinology and Metabolism* **51**, 674-676

DeNoto F.M., Moore D.D. and Goodman P. (1981) Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. *Nucleic Acid Research* **9**, 3719-3730

DeVroede M.A., Tseng L.Y-H., Katsoyannis P.G., Nissley S.P. and Rechler M.M. (1986) Modulation of insulin-like growth factor I binding to human fibroblast monolayer cultures by insulin-like growth factor carrier proteins released to the incubation media. *Journal of Clinical Investigation* **77**, 602-613

Dickson M.C., Saunders J.C. and Gilmour R.S. (1991) The ovine insulin-like growth factor-I gene: characterization, expression and identification of a putative promoter. *Journal of Molecular Endocrinology* **6,** 17-31

Dimitriadis G., Parry-Billings M., Dunger D., Bevan S., Colquhoun A., Taylor A., Calder P., Krause U., Wegener G. and Newsholme E.A. (1992) Effects of *in-vivo* administration of insulin-like growth factor-I on the rate of glucose utilization in the soleus muscle of the rat. *Journal of Endocrinology* **133**, 37-43

Domenè H., Krishnamurthi K., Eshet R., Gilad I., Laron Z., Koch I., Stannard B., Cassorla F., Roberts C.T. and LeRoith D. (1993) Growth hormone (GH) stimulates insulin-like growth factor-I (IGF-I) and IGF-I-binding protein-3, but not GH receptor gene expression in livers of juvenile rats. *Endocrinology* **133**, 675-682

Domenè H.M., Meidan R., Yakar S., Shen-orr Z., Cassorla F., Roberts C.T. Jr. and LeRoith D. (1994) Role of GH and IGF-I in the regulation of IGF-I, IGF-I receptor and IGF binding protein gene expression in the rat spleen. *Regulatory Peptides* **52**, 215-226

Donovan S.M., Oh Y., Pham H. and Rosenfeld R.G. (1989) Ontogeny of serum insulin-like growth factor binding proteins in the rat. *Endocrinology* **125**, 2621-2627

Drop S.L.S., Brinkman A., Kortleve D.J., Groffen C.A.H., Schuller A. and Zwarthoff E.C. (1991) The evolution of the insulin-like growth factor binding protein family. *In Modern concepts of insulin-like growth factors*. pp 311-328 Ed. E.M. Spencer Elsevier Science Publishing Co. Inc., Amsterdam

Drop S.L.S., Kortleve D.J., Guyda H.J. and Posner B.I. (1984) Immunoassay of a somatomedin-binding protein from human amniotic fluid: levels in fetal, neonatal and adult sera. *Journal of Clinical Endocrinology and Metabolism* **59**, 908-915

Dulak N.C. and Temin H.M. (1973) Multiplication-stimulating activity for chicken embryo fibroblasts from rat liver cell conditioned medium: a family of small polypeptides. *Journal of Cellular Physiology* **81,** 161-170

Duncan J.R. and Kornfeld S. (1988) Intracellular movement of two mannose-6-phosphate receptors: return to the Golgi apparatus. *Journal of Cell Biology* **106**, 617-628

Dunger D.B., Edge J.A., Pal R., Taylor A.M., Holly J.M. and Matthews D.R. (1991) The impact of increased growth hormone secretion on carbohydrate metabolism in adolescents with diabetes. *Acta Paediatrica Scandinavia* **377**, 69-78

Dunshea F.R., Bauman D.E., Boyd R.D. and Bell A.W. (1992b) Temporal response of circulating metabolites and hormones during somatotropin treatment of growing pigs. *Journal of Animal Science* **70**, 123-131

Dunshea F.R., Harris D.M., Bauman D.E., Boyd R.D. and Bell A.W. (1992a) Effect of porcine somatotropin on in vivo glucose kinetics and lipogenesis in growing pigs. *Journal of Animal Science* **70**, 141-151

Dvorák B., Stephana A.L., Holubec H., Williams C.S., Phillips A.F. and Koldovskoý O. (1996) Insulin-like growth factor-I (IGF-I) mRNA in the small intestine of suckling and adult rats. *FEBS Letters* **388**, 155-160

Elford P.R. and Lambert S.W.J. (1990) Contrasting modulation by transforming growth factor-β-1 of insulin-like growth factor-I production in osteoblasts and chondrocytes. *Endocrinology* **127**, 1635-1639

Elgin R.G., Busby W.H. and Clemmons D.R. (1987) An insulin-like growth factor (IGF) binding protein enhances the biological response to IGF-I. *Proceedings of the National Academy of Sciences USA* **84**, 3254-3258

El Nahas A.M., Le Carpentier J.E., Bassett A.H. and Hill D.J. (1989) Dietary protein and insulin-like growth factor-I content following unilateral nephrectomy. *Kidney International* **27,** S15-S19

Engberg G. and Hall K. (1984) Immunoreactive IGF-II in serum of healthy subjects and patients with growth hormone disturbances and uraemia. *Acta Endocrinologica* **107**, 164-170

Etherton, T.D., Wiggins J.P., Chung C.S., Evock C.M. Rebhun J.F., and Walton P.E. (1986) Stimulation of pig growth performance by porcine growth hormone and growth hormone releasing factor. *Journal of Animal Science* **63** 1389

Evock C.M., Etherton T.D., Chung C.S. and Ivy R.E. (1988) Pituitary porcine growth hormone (pGH) and recombinant pGH analog stimulate pig growth performance in a similar manner. *Journal of Animal Science* **66**, 1928-1941.

Evock C.M., Walton P.E. and Etherton T.D. (1990) Effect of GH status on IGF-I and IGF-II concentrations and serum IGF binding profiles in pigs. *Journal of Animal Science* **68**, 1953-1964

Ewton D.Z. and Florini J.R. (1981) Effects of somatomedins and insulin on myoblast differentiation *in vitro. Developmental Biology* **56**, 31-39

Feld S. and Hirschberg R. (1996) Insulin like growth factor-I and the kidney. *Trends in Endocrinology and Metabolism* **7**, 85-93

Fielder P.J., Gargosky S.E., Vaccarello M., Wilson K., Cohen P., Diamond F., Guevara-Aguirre J., Rosenbloom A.L. and Rosenfeld R.G. (1993) Serum profiles of insulin-like growth factors and their binding proteins in adults with growth hormone receptor deficiency treated with insulin-like growth factor I. *Acta Paediatrica* Suppl. **388**, 40-43

Fielder P.J., Guevara-Aguirre J., Rosenbloom A.L., Carlsson L., Hintz R.L. and Rosenfeld R.G. (1992) Expression of serum insulin-like growth factors (IGFs), insulin-like growth factor binding proteins (IGFBPs), and growth hormone binding protein (GHBP) in heterozygote relatives of Ecuadorian GH-receptor deficient patients. *Journal of Clinical Endocrinology and Metabolism* **74**, 743-750

Fielder P.J., Mortensen D.L., Mallet P., Carlsson B., Baxter R.C. and Clark R.G. (1996) Differential long-term effects of insulin-like growth factor-I (IGF-I), growth hormone (GH), and IGF-I plus GH on body growth and IGF binding proteins in hypophysectomized rats. *Endocrinology* **137**, 1913-1920

Fletcher T.P., Thomas G.B., Dunshea F.R., Moore L.G. and Clarke I.J. (1995) IGF feedback effects on growth hormone secretion in ewes: evidence for action at the pituitary but not the hypothalamic level. *Journal of Endocrinology* **144** 323-331

Florini J.R., Nicholson M.L. and Dulak N.C. (1977) Effects of peptide anabolic hormones on growth of myoblasts in culture. *Endocrinology* **101**, 32-41

Flyvbjerg A., Nielsen S., Sheikh M.I., Jacobsen C., Orskov H. and Christensen E.I. (1993) Luminal and basolateral uptake and receptor binding of IGF-I in rabbit renal proximal tubules. *American Journal of Physiology* **265**, F624-F633

Flyvbjerg A., Orskov H., Nyborg K Frystyk J., Marshall S.M., Bornfeldt K.B., Arnquist H.J. and Jorgensen K.D. (1991) Kidney IGF-I accumulation occurs in four different conditions with rapid initial kidney growth in rats. In *Modern Concepts of Insulin-like Growth Factors* pp 207-217 Ed. E.M. Spencer Elsevier Publishing Co. Inc., Amsterdam

Foley T.D., DePhillip R., Perricelli A. and Miller A. (1980) Low somatomedin activity in cord serum from infants with intrauterine growth retardation. *Journal of Paediatrics* **96**, 603-610

Fotsis T. Murphy C. and Gannon F. (1990) Nucleotide sequence of the bovine insulin-like growth factor I (IGF-I) and its IGF-IA precursor. *Nucleic Acids Res.* **3.** 676-677

Francis G.L., McNamara P.J., Filsell O.M. and Ballard F.J. (1988) Plasma half-lives of native and modified insulin-like growth factor-I in lambs. *Journal of Endocrinology* **117**, 183-189

Francis G.L., Read L.C., Ballard F.J., Bagley C.J., Upton F.M., Gravestock P.M. and Wallace J.C. (1986) Purification and partial sequence analysis of insulin-like growth factor-I from bovine colostrum. *Biochemical Journal* **233**, 207-213

Francis G.L., Ross M., Ballard F.J., Milner S.J., Senn C., McNeil K.A., Wallace J.C., King R. and Wells J.R.E. (1992) Novel recombinant fusion protein analogues of insulin-like growth factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for enhanced biological potency. *Journal of Molecular Endocrinology* **8**, 213-223

Fukagawa N.K., Minaker K.L., Rowe J.W., Goodman M.N., Matthews D.E., Bier D.M. and Young V.R. (1985) Insulin-mediated reduction of whole body protein breakdown. Dose-response effects on leucine metabolism in postabsorptive men. *Journal of Clinical Investigation* **76**, 2306-2311

Gargosky S.E., Moyse K.J., Walton P.E., Owens J.A., Wallace J.C., Robinson J.S. and Owens P.C. (1990) Circulating levels of insulin-like growth factors increase and molecular forms of their binding proteins change with human pregnancy. *Biochemical and Biophysical Research Communications* **170**, 1157-1163

Gargosky S.E., Pham H.M., Wilson K.F., Liu F., Giudice L.C. and Rosenfeld R.G. (1992) Measurement and characterization of insulin-like growth factor binding protein-3 in human biological fluids: discrepancies between radioimmunoassay and ligand blotting *Endocrinology* **131**, 3051-3060

Gaskins H.R., Jong-Wong K., Wright J.T., Rund L.A. and Hausman G.J. (1990) Regulation of insulin-like growth factor-I ribonucleic acid expression, polypeptide secretion, and binding protein activity by growth hormone in porcine preadipocyte cultures. *Endocrinology* **126**, 622-630

Geier A., Haimshon M., Beery R., Hemi R. and Lunenfeld B. (1993) Insulin-like growth factor-I inhibits cell death induced by cyclohexamide in MCF-7 cells: a model system for analyzing control of cell death. *In Vitro Cellular and Developmental Biology* **28 A**, 725-729

Glasscock G.F., Gelber S.E., Lamson G., McGee-Tekula R. and Rosenfeld R.G. (1990) Pituitary control of growth in the neonatal rat; effects of neonatal hypophysectomy on

somatic and organ growth, serum insulin like growth factors IGF-I and II levels and the expression of IGF binding proteins. *Endocrinology* **127**, 1792-1803

Glasscock G.F., Gin K.K.L., Kim J.D., Hintz R.L. and Rosenfeld R.G. (1991) Ontogeny of pituitary regulation of growth in the developing rat: comparison of effects of hypophysectomy and hormone replacement on somatic and organ growth, serum insulin-like growth factor-I (IGF-I) and IGF-II levels, and IGF-binding protein levels in the neonatal and juvenile rat. *Endocrinology* **128**, 1036-1047

Glasscock G.F., Hein A.N., Miller J.A., Hintz R.L. and Rosenfeld R.G. (1992) Effects of continuous infusion of insulin-like growth factor I and II, alone and in combination with thyroxine or growth hormone, on neonatal hypophysectomized rat. *Endocrinology* **130**, 203-210

Gluckman P.D. and Brinsmead M.W. (1976) Somatomedin in cord blood: relationship to gestational age and birth size. *Journal of Clinical Endocrinology and Metabolism* **43**, 1378-1381

Gluckman P.D. and Butler J.H. (1983) Parturition-related changes in insulin-like growth factor-I and -II in the perinatal lamb. *Journal of Endocrinolgy* **99**, 223-232

Goodband R.D., Hines R.H., Nelssen J.L., Kropf D.H. and Schricker B.R. (1993) Porcine somatotropin and dietary lysine influence bone mineralization and mechanical properties of bones in finishing swine. *Journal of Animal Science* **71**, 673-678

Goodyer C.G., DeStephano L., Lai W.H., and Posner B.I. (1984) Characterization of insulin-like growth factor receptors in rat anterior pituitary, hypothalamus and brain. *Endocrinology* **114**, 1187-1195

Gopinath R. and Etherton T.D. (1989a) Effects of porcine growth hormone on glucose metabolism of pigs: I. Acute and chronic effects on plasma glucose and insulin status. *Journal of Animal Science* **67**, 682-688

Gopinath R. and Etherton T.D. (1989b) Effects of porcine growth hormone on glucose metabolism of pigs: II Glucose tolerance, peripheral tissue insulin sensitivity and glucose kinetics. *Journal of Animal Science* **67**, 689-697

Gopinath R., Walton P.E. and Etherton T.D. (1989) An acid-stable insulin-like growth factor (IGF)-binding protein from pig serum inhibits binding of IGF-I and IGF-II to vascular endothelial cells. *Journal of Endocrinology* **120**, 231-236.

Gosteli-Peter M.A., Winterhalter K.H., Schmid C., Froesch E.R. and Zapf J. (1994) Expression and regulation of insulin-like growth factor-I (IGF-I) and IGF-binding protein messenger ribonucleic acid levels in tissues of hypophysectomized rats infused with IGF-I and growth hormone. *Endocrinology* **135**, 2558-2567

Grant A.L., Helferich W.G., Kramer S.A., Merkel R.A. and Bergen W.G. (1991) Administration of growth hormone to pigs alters the relative amount of insulin-like growth factor-I mRNA in liver and skeletal muscle. *Journal of Endocrinology* **130**, 331-338

Grimes R.W. and Hammond J.M. (1992) Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-binding proteins by ovarian granulosa cells. *Endocrinology* **131**, 553-558

Guler H.P., Schmid C., Zapf J. and Froesch E.R. (1989) Effects of recombinant insulin-like growth factor I on insulin secretion and renal function in normal human subjects. *Proceedings of the National Academy of Science, USA* **86**, 22868-2872

Guler H.P., Zapf J. and Froesch E.R. (1987) Short-term metabolic effects of recombinant human insulin-like growth factor-I in healthy adults. *New England Journal of Medicine* **317**, 137-140

Guler H.P., Zapf J., Scheiwiller E. and Foesch E.R. (1988) Recombinant human insulin-like growth factor I stimulates growth and has distinct effects on organ size in hypophysectomized rats. *Proceedings of the National Academy of Science, USA* **85**, 4889-4893

Gustafson T.A. and Rutter W.J. (1990) The cysteine-rich domains of the insulin-like growth factor I receptors are primary determinants of hormone binding specificity: evidence from receptor chimeras. *Journal of Biological Chemistry* **265**, 18663-18667

Hall K., Hansson U., Lundin G., Luthman M., Persson B., Povoa G., Stagenberg M. and Ofverholm U. (1986) Serum levels of somatomedins and somatomedin binding protein in pregnant women with type I or gestational diabetes and their infants. *Journal of Clinical Endocrinology and Metabolism* **63**, 1300-1306

Hall K., Lundin G. and Povoa G. (1988) Serum levels of the low molecular weight form of insulin-like growth factor binding protein in healthy subjects and patients with growth hormone deficiency, acromegaly and anorexia nervosa. *Acta Endocrinologica* **118**, 321-326

Hall K. and Sara V.R. (1984) Somatomedin levels in childhood, adolescence and in adult life. *Clinics in Endocrinology and Metabolism* **13**, 91-112

Hall K. and Uthne K. (1971) Some biological properties of purified sulfation factor (SF) from human plasma. *Acta Med. Scand.* **190,** 137-143

Han V.K.M, Asano H., Matsell D.G., Delhanty P.J.D., Hayatsu J., Bassett N., Phillips I.D., Nygard K. and Stepaniuk O. (1994) Insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) in fetal development: physiology and pathophysiology. In *The insulin-*

like growth factors and their regulatory proteins. pp 263-274 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld Elsevier Science Publishing Co. Inc., Amsterdam

Han V.K.M., Lauder J.M. and D'Ercole A.J. (1987) Characterization of somatomedin/insulin-like growth factor receptors and correlation with biological action in cultured neonatal rat astroglial cells. *Journal of Neuroscience* **7**, 501-511

Han V.K.M., Lund P.K., Lee D.C. and D'Ercole A.J. (1988) Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization and tissue distribution. *Journal of Clinical Endocrinology and Metabolism* **66**, 422-429

Hansen I. Tsalikian E., Beaufrere B., Gerich J., Haymond M. and Rizza R. (1986) Insulin resistance in acromegaly: Defects in both hepatic and extrahepatic insulin action. *American Journal of Physiology* **250**, E269-E273

Harrington E.A., Bennett M.R., Fanidi A. and Evan G.I. (1994) c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO Journal* **13**, 3286-3295

Hazel S.J., Gillespie C.M., Moore R.J., Clark R.G., Jureidini K.F. and Martin A.A. (1994) Enhanced body growth in uremic rats treated with IGF-I and growth hormone in combination. *Kidney International* **46**, 58-68

Hilding A., Brismar K., Thorén M. and Hall K. (1993) Glucagon stimulates insulin-like growth factor binding protein -1(IGFBP-1) in healthy subjects, patients with pituitary insufficiency and patients with insulin dependent diabetes mellitus *Journal of Clinical Endocrinology and Metabolism* **77**, 1142-1147

Hill D.J. (1979) Stimulation of cartilage zones of the calf costo-chondral growth plate *in vitro* by growth hormone dependent rat plasma somatomedin activity. *Journal of Endocrinology* **83**, 219-227

Hill D.J., Crace C.J., Strain A.J. and Milner R.D.G. (1986) Regulation of amino acid uptake and DNA synthesis in isolated human fetal fibroblasts and myoblasts: Effect of human placental lactogen, somatomedin-C, multiplication-stimulating activity and insulin. *Journal of Clinical Endocrinology and Metabolism* **62**, 753-760

Hirschberg R. and Kopple J.D. (1988) Increase in renal plasma flow and glomerular filtration rate during growth hormone treatment may be mediated by insulin-like growth factor I. *American Journal of Nephrology* **8**, 249-254

Hirschberg R. and Kopple J.D. (1989) Evidence that insulin-like growth factor I increases renal plasma flow and glomerular filtration rate in fasted rats. *Journal of Clinical Investigation* **83**, 326-330

Hirschberg R., Brunori G., Kopple J.D. and Guler H.P. (1993) Effects of insulin-like growth factor I on renal function in normal men. *Kidney International* **43**, 387-397

Hirschberg R., Rabb H., Bergamo R. and Kopple J.D. (1989) The delayed effect of GH on renal function in humans. *Kidney International* **35**, 865-870

Hizuka N., Takano K., Shizume K., Asakawa K., Miyakawa M., Tanaka I. and Horikawa R. (1986) Insulin-like growth factor I stimulates growth in normal growing rats. *European Journal of Pharmacology* **125**, 143- 146

Hock J.M., Centrella M. and Canalis E. (1988) Insulin-like growth factor I has dependent effects on bone matrix formation and cell replication. *Endocrinology* **122**, 254-260

Hodgkinson S.C., Napier J.R., Spencer G.S.G. and Bass J.J. (1994) Glycosaminoglycan binding characteristics of the insulin-like growth factor binding proteins. *Journal of Molecular Endocrinology* **13**, 105-112

Holly J.M.P., Coulson V.J., Davies S.C., Cotterill A.M., *et al.* (1993) The presence of a circulating enzyme which specifically modifies the main carrier protein for insulin-like growth factors (IGFs) in GH-deficient and GH-resistant subjects. *Journal of Endocrinology (Suppl)* **131**, Abstract 8

Holthuizen P.E., Rodenburg R.J.T., Scheper W. and Sussenbach J.S. (1994) Regulation of IGF-II gene expression and posttranscriptional processing of IGF-II mRNAs. In *The Insulin-like growth factors and their regulatory proteins*. pp 43-53 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld Elsevier Science Publishing Co. Inc., Amsterdam

Hossenlopp P., Sergovia B., Lassare C., Roghani M., Bredon M. and Binoux M. (1990) Evidence of enzymatic degradation of insulin-like growth factor binding proteins (IGFBPs) in the 150 K complex during pregnancy. *Journal of Clinical Endocrinology and Metabolism* **71**, 797-805

Hoyt E.C., Van Wyk J.J. and Lund P.K. (1988) Tissue and development specific regulation of a complex family of rat insulin-like growth factor I messenger ribonucleic acids. *Molecular Endocrinology* **2**, 1077-1086

Hsu C.J. and Hammond J.M. (1987) Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells in vitro. *Endocrinology* **120,** 198-207

Hua K.M., Ord R., Kirk S., Li Q.J., Hodgkinson S.C., Spencer G.S.G., Molan P.C. and Bass J.J. (1993) Regulation of plasma and tissue levels of insulin-like growth factor-I by nutrition and treatment with growth hormone in sheep. *Journal of Endocrinology* **136** 217-224

Hussain M.A., Schmitz O., Mengel A., Keller A., Christiansen J.S., Zapf J. and Froesch E.R. (1994) Comparison of the effects of growth hormone and insulin-like growth factor-I in growth hormone-deficient subjects. *Journal of Clinical Investigation* **94**, 1126-1133

Isaksson O.G., Jansson J.O. and Gause I.A.M. (1982) Growth hormone stimulates longitudinal bone growth directly. *Science* **216**, 1237-1239

Isgaard J., Nilsson A., Vikman K. and Isaksson O.G.P. (1989) Growth hormone regulates the level of insulin-like growth factor-I mRNA in rat skeletal muscle. *Journal of Endocrinology* **120**, 107-112

Jacobs S. and Cuatrecasas P. (1986) Phosphorylation of receptors for insulin and insulin-like growth factor I: effects of hormones and phorbol esters. *Journal of Biological Chemistry* **261**, 934-939

Jansen E., Steenbergh P.H., Le Roith D., Roberts C.T. and Sussenbach J.S. (1991) Identification of multiple transcription start sites in the human insulin-like growth factor-I gene. *Molecular and Cellular Endocrinology* **78** 115-125

Jansen E., Steenbergh P.H., Van Schaik F.M.A. and Sussenbach J.S. (1992) The human IGF-I gene contains two cell type-specifically regulated promoters. *Biochemical and Biophysical Research Communications* **187**, 1219-1226

Jansen E., Van Schaik F.M.A., Ricker A.T. Bullock B., Woods D.E., Gabbay K.H. Nussbaum A.L., Sussenbach J.S. and van den Brande J.L. (1983) Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature* **306**, 609-611

Jansen E., Van Schaik F.M.A., van Tol H., Van den Brande J.L. and Sussenbach J.S. (1985) Nucleotide sequence of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II and an IGF-II variant). *FEBS Letters* **179**, 243-246

Jewell D.E., Hausman G.J. and Campion D.R. (1989) Fetal hypophysectomy causes a decrease in preadipocyte growth and insulin-like growth factor-I in pigs. *Domestic Animal Endocrinology* **6** 243-252

Johnson J.L., Coffey M.T., Esbenshade K.L., Schricker B.R. and Pilkington D.H. (1990) The effect of human growth hormone-releasing factor or porcine somatotropin on serum hormones and metabolites, growth performance and carcass traits in swine. *Journal of Animal Science* **68**, 3204-3211

Jones J.I., Busby W.H., Wright G., Smith C.E., Kimack N.M. and Clemmons D.R. (1993) Identification of the sites of phosphorylation in insulin-like growth factor binding protein-1: Regulation of its affinity by phosphorylation of serine 101. *Journal of Biological Chemistry* **268**, 1125-1131

Jorgensen J.O.L., Blum W.F., Moller N., Ranke M.B. and Christiansen J.S. (1991) Short-term changes in serum insulin-like growth factors (IGF) and IGF binding protein 3 after different modes of intravenous growth hormone (GH) exposure in GH-deficient patients. *Journal of Clinical Endocrinology and Metabolism* **72**, 582-587

Julkunen M., Koistinen R., Aalto-Setälä K. Seppala M., Janne O.A. and Kontula K. (1988) Primary structure of human insulin-like growth factor binding protein/placental protein 12 and tissue specific expression of its mRNA. *FEBS Letters* **236**, 295-302

Jyung R.W., Mustoe T., Busby W.H. and Clemmons D.R. (1994) Increased wound breaking strength induced by insulin-like growth factor-I in combination with insulin-like growth factor binding protein-1. *Surgery* **115**, 233-239

Kajimoto Y. and Rotwein P. (1991) Structure of the chicken insulin-like growth factor I gene reveals conserved promoter elements. *Journal of Biological Chemistry* **266,** 9724-9731

Kampman K.A., Ramsay T.G. and White M.E. (1993) Developmental changes in IGF-2 mRNA levels in intrauterine growth retarded and control swine *Comparative Biochemistry and Physiology* **104**, 415-421

Kanety H., Karasik A., Klinger B., Silbergeld A. and Laron Z. (1993) Long-term treatment of Laron type dwarfs with insulin-like growth factor-1 increases serum insulin-like growth factor-binding protein-3 in the absence of growth hormone activity. *Acta Endocrinologica* **128**, 144-149

Katakami H., Arimura A. and Frohman L.A. (1986) Growth hormone (GH)-releasing factor stimulates hypothalamic somatostatin release: an inhibitory feedback effect on GH secretion. *Endocrinology* **118**, 1872-1877

Kavsan V.M., Koval A.P., Grebenjuk V.A., Chan S.J., Steiner D.F., Roberts, Jr. C.T. and LeRoith D. (1993) Structure of the chum salmon insulin-like growth factor-I gene. *DNA and Cell Biology* **12**, 729-737

Kazumi T., Vranic M., Bar On H. and Steiner G. (1986) Portal *versus* peripheral hyperinsulinemia and very low density lipoprotein triglyceride kinetics. *Metabolism* **35**, 1024-1028

Kiess W., Blickenstaff G.D., Sklar M.M. Thomas C.L., Nissley S.P. and Sahagian G.G. (1988) Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. *Journal of Biological Chemistry* **263**, 9339-9344

Kiess W., Greenstein L.A., White R.M., Lee L., Rechler M.M. and Nissley S.P. (1987) The type II insulin-like growth factor is present in rat serum. *Proceedings of the National Academy of Sciences, USA* **84**,7720-7724

King R., Wells J.R.E., Krieg P., Snoswell M., Brazier J., Bagley C.J., Wallace J.C.W., Ballard F.J., Ross M. and Francis G.L. (1992) Production and characterization of recombinant insulin-like growth factor-I (IGF-I) and potent analogues of IGF-I, with Gly or Arg substituted for Glu^3, following their expression in *Escherichia coli* as fusion proteins. *Journal of Molecular Endocrinology* **8**, 29-41

Klapper D.G., Svoboda M.E. and Van Wyk J.J. (1983) Sequence analysis of Somatomedin-C: confirmation of identity with insulin-like growth factor-I. *Endocrinology* **112,** 2215-2217

Klindt J., Buonomo F.C. and Yen J.T. (1992) Administration of porcine somatotropin by sustained-release implant: Growth and endocrine responses in genetically lean and obese barrows and gilts. *Journal of Animal Science* **70**, 3721-3733

Kupfer S.R., Underwood L.E., Baxter R.C. and Clemmons D.R. (1993) Enhancement of the anabolic effects of growth hormone and insulin-like growth factor I by use of both agents simultaneously. *Journal of Clinical Investigation* **91**, 391-396

Lajara R., Rotwein P., Bortz J.D., Hansen V.A., Sadow J.L., Betts C.R., Rogers S.A. and Hammerman M.R. (1989) Dual regulation of insulin-like growth factor I expression during renal hypertrophy. *American Journal of Physiology* **257**, F252-F261

Lamson G., Oh Y., Pham H., Giudice L.C. and Rosenfeld R.G. (1989) Expression of two insulin-like growth factor-binding proteins in a human endometrial cancer cell line: structural, immunological and genetic characterization. *Journal of Clinical Endocrinology and Metabolism* **69**, 852-859

Lamson G., Giudice L.C. and Rosenfeld R.G. (1991) A simple assay for proteolysis of IGFBP-3. *Journal of Clinical Endocrinology and Metabolism* **72**, 1391-1393

Lanzi R. and Tannenbaum G.S. (1992) Time course and mechanism of growth hormone's negative feedback effect on its own spontaneous release. *Endocrinology* **130**, 780-788

Laron Z. (1993) Somatomedin-1 (Insulin-like growth factor-I) in clinical use. Facts and potential. *Drugs* **45**, 1-8

Laron Z., Anin S., Klipper-Aurbach Y. and Klinger B. (1992) Effects of insulin-like growth factor on linear growth, head circumference, and body fat in patients with Laron-type dwarfism. *Lancet* **339**, 1258-1261

Laron Z., Pertzelan A., Karp M., Kowadlo-Silbergeld A. and Daughaday W.H. (1971) Administration of growth hormone to patients with familial dwarfism with high plasma immunoreactive growth hormone. Measurements of sulfation factor, metabolic and linear growth responses. *Journal of Clinical Endocrinology and Metabolism* **33**, 332-342

Lassarre C., Lalou C., Perin L. and Binoux M. (1994) Protease induced alteration of insulin-like growth factor binding protein-3 as detected by radioimmunoassay. Agreement with ligand blotting data. *Growth Regulation* **4**, 48-55

Leahy J.L. and Vandekerkhove K.M. (1990) Insulin-like growth factor-I at physiological concentrations is a potent inhibitor of insulin secretion. *Endocrinology* **126**, 1593-1598

Lee C.Y., Bazer F.W., Etherton T.D. and Simmen F.A. (1991) Ontogeny of insulin-like growth factors (IGF-I and IGF-II) and IGF binding proteins in porcine serum during fetal and postnatal development. *Endocrinology* **128** 2336-2344

Lee C.Y., Chung C.S. and Simmen F.A. (1993b) Ontogeny of the porcine insulin-like growth factor system. *Molecular and Cellular Endocrinology* **93**, 71-80

Lee C.Y., Simmen F.A. and Bazer F.W. (1990) Serum concentrations of insulin-like growth factors I and II during fetal and postnatal development of pigs. *Journal of Animal Science* **68** (Suppl 1), 309

Lee P.D.K., Jensen M.D., Divertie G.D., Heiling V.J., Katz H.H. and Conover C.A. (1993a) Insulin-like growth factor binding protein-1 response to insulin during suppression of endogenous insulin secretion. *Metabolism* **42**, 409-414

Lefaucheur L., Missohou A., Ecolan P., Monin G. and Bonneau M. (1992) Performance, plasma hormones, histochemical and biochemical muscle traits, and meat quality of pigs administered exogenous somatotropin between 30 or 60 kilograms and 100 kilograms body weight. *Journal of Animal Science* **70**, 3401-3411

Lemmey A.B., Martin A.A., Read L.C., Tomas F.M., Owens P.C. and Ballard F.J. (1991) IGF-I and the truncated analogue des(1-3)IGF-I enhance growth in rats after gut resection. *American Journal of Physiology* **260**, E213-E219

Lemozy S., Pucilowska J.B and Underwood L.E. (1994) Reduction of insulin-like growth factor-I (IGF-I) in protein-restricted rats is associated with differential regulation of IGF-binding protein messenger ribonucleic acids in liver and kidney, and peptides in liver and serum. *Endocrinology* **135**, 617-623

Leonards J.R. and Landau B.R. (1962) Assay of insulin and insulin-like activity with rat epididymal fat pad. *Journal of Laboratory and Clinical Medicine* **60,** 552-570

Leong S.R., Baxter R.C., Camerato T., Dai J. and Wood W.I. (1992) Structure and functional expression of the acid-labile subunit of the insulin-like growth factor-binding protein complex. *Molecular Endocrinology* **6**, 870-876

LeRoith D., Werner H., Burguera B., Roberts C.T., Mulroney S. and Haramati A. (1992) The insulin-like growth factor family of peptides, binding proteins and receptors: their

potential role in tissue regeneration. In *Pancreatic Islet Cell Regeneration and Growth* pp 21-28 Ed. A.I. Vinik Plenum Press, New York

Le Stunff C., Thomas M.J. and Rotwein P. (1995) Rapid activation of rat insulin-like growth factor-I gene transcription by growth hormone reveals no changes in deoxyribonucleic acid-protein interactions within the second promoter. *Endocrinology* **136**, 2230-2237

Lewitt M.S. and Baxter R.C. (1990) Inhibitors of glucose uptake stimulate the production of insulin-like growth factor binding protein-1 (IGFBP-1) by human fetal liver. *Endocrinology* **126**, 1527-1533

Lewitt M.S., Saunders H. and Baxter R.C. (1992) Regulation of rat insulin-like growth factor-binding protein-1: The effect of insulin-induced hypoglycemia. *Endocrinology* **131**, 2357-2364

Lewitt M.S., Saunders G.J., Cooney G.J. and Baxter R.C. (1993) Effect of human insulin-like growth factor-binding protein-1 on the half life and action of administrated insulin-like growth factor-I in rats. *Journal of Endocrinology* **136**, 253-260

Lewitt M.S., Saunders H., Phuyal J.L. and Baxter R.C. (1994) Complex formation by human insulin-like growth factor-binding protein-3 and human acid-labile subunit in growth hormone-deficient rats. *Endocrinology* **134**, 2404-2409

Lin T., Haskell J., Vinson N. and Terracio L. (1986) Characterization of insulin and insulin-like growth factor I receptors of purified Leydig cells and their role in steroidogenesis in primary cultures: a comparative study. *Endocrinology* **119**, 1641-1647

Liu F., Powell D.R. and Hintz R.L. (1990) Characterization of insulin-like growth factor binding proteins in human serum from patients with chronic renal failure. *Journal of Clinical Endocrinology and Metabolism* **70**, 620-628

Liu J., Baker J., Perkins A.S. Robertson E.J. and Efstratiadis A. (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (IGF-I) and type 1 IGF receptor. *Cell* **75**, 59-72

Liu L.L., Greenberg S., Russell S.M. and Nicoll C.S. (1989) Effects of insulin-like growth factors I and II on growth and differentiation of transplanted rat embryos and fetal tissues. *Endocrinology* **124**, 3077-3082

Lo H.C., Hinton P.S., Peterson C.A. and Ney D.M. (1995) Simultaneous treatment with IGF-I and GH additively increases anabolism in parenterally fed rats. *American Journal of Physiology* **269**, E368-E376

Lowe W.L. Jr., Lasky S.R., LeRoith D. and Roberts C.T. Jr. (1988) Distribution and regulation of rat insulin-like growth factor I messenger ribonucleic acids encoding alternative carboxyterminal E-peptides: evidence for differential processing and regulation in liver. *Molecular Endocrinology* **2**, 528-535

Luo J. and Murphy L.J. (1989) Dexamethasone inhibits growth hormone induction of insulin-like growth factor-I (IGF-I) messenger ribonucleic acid (mRNA) in hypophysectomized rats and reduces IGF-I mRNA abundance in the intact rat. *Endocrinology* **125**, 165-171

McClain D.A. Maegawa H., Levy J., Hueckstead T., Dull T.J., Lee J., Ullrich A. and Olefsky J.M. (1988) Properties of a human insulin receptor with a COOH-terminal truncation. I. Insulin binding, autophosphorylation, and endocytosis. *Journal of Biological Chemistry* **263**, 8904-8911

McCusker R.H., Camacho-Hubner C., Baynes M.L., Cascieri M.A. and Clemmons D.R. (1990) Insulin-like growth factor (IGF) binding to human fibroblast and glioblastoma cells: the modulating effect of cell released IGF binding proteins. *Journal of Cellular Physiology* **144**, 244-253

McCusker R.H., Campion D.R. and Clemmons D.R. (1988) The ontogeny and regulation of a 31 000 M_r insulin-like growth factor/somatomedin (IGF) binding protein in fetal porcine plasma and sera. *Endocrinology* **122**, 2071-2079

McCusker R.H., Campion D.R., Jones W.K. and Clemmons D.R. (1989) The insulin-like growth factor-binding proteins of porcine serum: Endocrine and nutritional regulation. *Endocrinology* **125**, 501-509

MacDonald R.G., Pfeffer S.R., Coussens L., Tepper M.A., Brocklebank C.M., Mole J.E., Anderson J.K., Chen E., Czech M.P. and Ullrich A. (1988) A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* **239**, 1134-1137

McMorris F.A. and Dubois-Dalcq M. (1988) Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing *in vitro*. *Journal of Neuroscientific Research* **21**, 199-209

McNamara J.P., Brekke C.J., Jones R.W. and Dalrymple R.H. (1991) Recombinant porcine somatotropin alters performance and carcass characteristics of heavyweight swine and swine fed alternative feedstuffs. *Journal of Animal Science* **69**, 2273-2281

Maes M., De Hertogh R., Watrin-Granger P. and Ketelslegers J.M. (1983) Ontogeny of liver somatotropic and lactogenic binding sites in male and female rats. *Endocrinology* **13**, 1325-1332

Maiter D., Walker J.L., Adam E., Moats-Staats B., Mulumba N., Ketelslegers J-M. and Underwood L.E. (1992) Differential regulation by growth hormone (GH) of insulin-like growth factor I and GH receptor/binding protein gene expression in rat liver. *Endocrinology* **130**, 3257-3264

Malmöf, K., Cortova Z., Saxerholt H., Karlsson E., Arrhenius-Nyberg V. and Skottner A. (1995) Effects of insulin-like growth factor-I and growth hormone on the net flux of amino acids across the hind limbs in the surgically traumatized pig. *Clinical Science* **88**, 285-292

Marcus R. Butterfield G., Holloway L., Gilliland L., Baylink D.J., Hintz R.L. and Sherman B.M. (1990) Effects of short term administration of recombinant human growth hormone to elderly people. *Journal of Clinical Endocrinology and Metabolism* **70**, 519-527

Marquart H. and Todaro G.J. (1981) Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. *Journal of Biological Chemistry* **256,** 6859-6865

Martial J.A., Baxter J.D., Goodman H.M. and Seeburg P.A. (1977) Regulation of growth hormone messenger RNA by thyroid and glucocorticoid hormones. *Proceedings of the National Academy of Science, USA* **74**, 1816-1820

Martin A.A., Tomas F.M., Owens P.C., Knowles S.E., Ballard F.J. and Read L.C. (1991) IGF-I and its variant, des-(1-3)IGF-I enhance growth in rats with reduced renal mass. *American Journal of Physiology* **261**, F626-F633

Mauras N. (1995) Combined recombinant human growth hormone and recombinant human insulin-like growth factor I: lack of synergy on whole body protein anabolism in normally fed subjects. *Journal of Clinical Endocrinology and Metabolism* **80,** 2633-2637

Matthews L.S., Hammer R.E., Behringer R.R., D'Ercole A.J., Bell G.I., Brinster R.L. and Palmiter R.D. (1988) Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* **126**, 1569-1575

Merriam G.R. and Wachter K.W. (1982) Algorithms for the study of episodic hormone secretion. *American Journal of Physiology* **243**, E310-E318

Mesiano S., Young I.R., Hey A.W., Browne C.A. and Thorburn G.D. (1989) Hypophysectomy of the fetal lamb leads to a fall in the plasma concentration of insulin-like growth factor I (IGF-I), but not IGF-II. *Endocrinology* **124**, 1485-1491

Michels K.M., Lee W., Seltzer A., Saverda J.M. and Bondy C.A. (1993) Up-regulation of pituitary ^{125}I insulin-like growth factor-I (IGF-I) binding and IGF binding protein-2 and IGF-I gene expression by estrogen. *Endocrinology* **132**, 23-29

Miell J.P., Taylor A.M., Jones J., Holly J.M.P. Gaillard R.C., Pralong F.P., Ross R.J.M. and Blum W.F. (1993a) The effects of dexamethasone treatment on immunoreactive and bioactive insulin-like growth factors (IGFs) and IGF-binding proteins in normal male volunteers. *Journal of Endocrinology* **136**, 525-533

Miell J.P., Taylor A.M., Zini M., Maheshwari H.G., Ross R.J.M. and Valcavi R. (1993b) Effects of hypothyroidism and hyperthyroidism on insulin-like growth factors (IGFs) and growth hormone- and IGF-binding proteins. *Journal of Clinical Endocrinology and Metabolism* **76**, 950-955

Min S.H., MacKenzie D.D.S., Breier B.H., McCutcheon S.N. and Gluckman P.D. (1996) Responses of young energy-restricted sheep to chronically administered insulin-like growth factor I (IGF-I): evidence that IGF-I suppresses the hepatic growth hormone receptor. *Endocrinology* **137**, 1129-1137

Minniti C.P., Kohn E.C., Grubb J.H., Sly W.S., Oh Y., Muller H.L., Rosenfeld R.G. and Helman L.J. (1992) The insulin-like growth factor II (IGF-II)/mannose-6-phosphate receptor mediates IGF-II induced motility in human rhabdomyosarcoma cells. *Journal of Biological Chemistry* **267**, 9000-9004

Mohan S., Bautista C.M., Wergedal J. and Baylink D.J. (1989) Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium: a potential regulator of IGF action. *Proceedings of the National Academy of Sciences, USA* **86**, 8338-8342

Mohan S., Strong D.D., Linkhart T.A. and Baylink D.J. (1994) Regulation and actions of insulin-like growth factor binding protein (IGFBP)-4 and IGFBP-5: physiological and clinical implications. In *The insulin-like growth factors and their regulatory proteins*. pp 205-215 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld Elsevier Science Publishing Co. Inc., Amsterdam

Mondschein J.S. and Hammond J.M. (1988) Growth factors regulate immunoreactive insulin-like growth factor-I production by cultured porcine granulosa cells. *Endocrinology* **123**, 463-468

Morell B. and Froesch E.R. (1973) Fibroblasts as an experimental tool in metabolic and hormone studies. II. Effects of insulin and nonsuppressible insulin-like activity (NSILAs) on fibroblasts in culture. *European Journal of Clinical Investigation* **3,** 119-123

Morgan C.J., Coutts A.G.P., McFadyen M.C., King T.P. and Kelly D. (1996) Characterization of IGF-I receptors in the porcine small intestine during postnatal development. *Nutritional Biochemistry* **7**, 339-347

Morgan D.O., Edman J.C., Standring D.N., Fried V.A., Smith M.C., Roth R.R. and Rutter W.J. (1987) Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* **329**, 301-307

Morgan J.M., Saris S.D., Capuzzi D.M., Good W.R., John V.A., Guler H-P., Seaman J. and Piraino A.J. (1993) Hypoglycemic and insulin response to a continuous intravenous infusion of CGP-35126 recombinant human insulin-like growth factor-I (rhIGF-I) in healthy males. *Journal of Clinical Pharmacology* **33**, 366-372

Moses A.C., Nissley S.P., Short P.A., Rechler M.M., White R.M., Knight A.B. and Higa O.Z. (1980) Elevated levels of multiplication-stimulating activity, an insulin-like growth factor, in fetal serum. *Proceedings of the National Academy of Sciences USA* **77**, 3649-3653

Moxham C. and Jacobs S. (1992) Insulin-like growth factor receptors. *In The Insulin-Like Growth Factors: Structure and Biological Functions.* pp 80-109 Ed. P.N. Schofield Oxford University Press, New York

Muller H.L., Oh Y., Gargosky S.E., Lehrnbecher T., Hintz R.L. and Rosenfeld R.G. (1993) Concentrations of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), IGF, and IGFBP-3 protease activity in cerebrospinal fluid of children with leukemia, central nervous system tumor, or meningitis. *Journal of Clinical Endocrinolgy and Metabolism* **77**, 1516-1521

Müller M. and Brem G. (1990) Nucleotide sequence of porcine insulin-like growth factor I: 5' untranslated region, exons 1 and 2 and mRNA. *Nucleic Acids Research* **18,** 364

Murphy L.J., Murphy L.C. and Friesen H.G. (1987) Estrogen induces insulin-like growth factor-I expression in the rat uterus. *Molecular Endocrinology* **1**, 445-450

Murphy W.R., Daughaday W.H. and Hartnett C. (1956) The effect of hypophysectomy and growth hormone on the incorporation of labelled sulfate into tibial epiphysial and nasal cartilage of the rat. *Journal of Laboratory and Clinical Medicine* **47,**715-722

Murray-Rust J., McLeod A.N., Blundell T.L. and Wood S.P. (1992) Structure and evolution of insulins: implications for receptor binding. *Bioessays* **14**, 325-331

Muta K. and Krantz (1993) Apoptosis of human erythroid colony forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythropoietin. *Journal of Cellular Physiology* **156**, 264-271

Nanto-Salonen K., Muller H.L., Hoffman A.R., Vu T.H. and Rosenfeld R.G. (1993) Mechanisms of thyroid hormone action on the insulin-like growth factor system: all thyroid hormone effects are not growth hormone mediated. *Endocrinology* **132**, 781-788

Nishimoto I., Hata Y., Ogata E. and Kojima I. (1987) Insulin-like growth factor II stimulates calcium influx in competent BALB/c 3T3 cells primed with epidermal growth factor. Characteristics of calcium influx and involvement of GTP-binding protein. *Journal of Biological Chemistry* **262**, 12120-12126

Nolten L.A., Van Schaik F.M.A., Steenbergh P.H. and Sussenbach J.S. (1994) Expression of the insulin-like growth factor I gene is stimulated by the liver-enriched transcription factors C/EBPα and LAP. *Molecular Endocrinology* **8**, 1636-1645

Nurjhan N., Campbell P.J., Kennedy F.P., Miles J.M. and Gerich J.E. (1986) Insulin dose-response characteristics for suppression of glycerol release and conversion to glucose in humans. *Diabetes* **36**, 1326-1331

O'Malley K.L. and Rotwein P. (1988) Human tyrosine hydroxylase and insulin genes are contiguous on chromosome 11. *Nucleic Acids Research* **16**, 4437-4446

Ooi G.T., Orlowski C.C., Brown A.L., Becker R.E., Unterman T.G. and Rechler M.M. (1990) Different tissue distribution and hormonal regulation of messenger RNAs encoding rat insulin-like growth factor-binding proteins-1 and -2. *Molecular Endocrinology* **4**, 321-328

Orlowski C.C., Brown A.L., Ooi G.T., Yang Y.W-H., Tseng L.Y-H. and Rechler M.M. (1990) Tissue, developmental and metabolic regulation of messenger ribonucleic acid encoding a rat insulin-like growth factor-binding protein. *Endocrinology* **126**, 644-652

Owens P.C., Conlon M.A., Campbell R.G., Johnson R.J., King R. and Ballard F.J. (1991) Developmental changes in growth hormone, insulin-like growth factors (IGF-I and IGF-II) and IGF-binding proteins in plasma of young growing pigs. *Journal of Endocrinology* **128**, 439-447

Owens P.C., Johnson R.J., Campbell R.G. and Ballard F.J. (1990) Growth hormone increases insulin-like growth factor-I (IGF-I) and decreases IGF-II in plasma of growing pigs. *Journal of Endocrinology* **124**, 269-275

Page G.S., Smith S. and Goodman H.M. (1981) DNA sequence of the rat growth hormone gene: location of the 5' terminus of the growth hormone mRNA and identification of an internal transposon-like element. *Nucleic Acid Research* **9**, 2087-2104

Palka J, Bird T.A., Oyamada I. and Peterkofsky B. (1989) Similar hormonal changes in sera from scorbutic and fasted (vitamin C-supplemented) guinea pigs, including decreased IGF-I and appearance of an IGF-I reversible mitogenic inhibitor. *Growth Factors* **1** 147-156

Palmer R.M., Flint D.J., MacRae J.C., Fairhurst F.E., Bruce L.A., Mackie S.C. and Lobley G.E. (1993) Effects of growth hormone and an antiserum to rat growth hormone on serum IGF-I and muscle protein synthesis and accretion in the rat. *Journal of Endocrinology* **139**, 395-401

Pao C.I., Farmer P.K., Begovic S., Villafuerte C., Wu G., Robertson D.G. and Phillips L.S. (1993) Regulation of insulin-like growth factor-I (IGF-I) and IGF-binding protein 1 gene transcription by hormones and provision of amino acids in rat hepatocytes. *Molecular Endocrinology* **7**, 1561-1568

Pardee A.B. (1989) G_1 events and regulation of cell proliferation. *Science* **246**, 603-608

Parving H., Noer I., Mogensen C and Svendsen P. (1978) Kidney function in normal man during short-term growth hormone infusion. *Acta Endocrinologica (Copenhagen)* **89**, 796-800

Pekonen F., Suikkari A.M., Makinen T. and Rutanen E-M. (1988) Different insulin-like growth factor binding species in human placenta and decidua. *Journal of Clinical Endocrinology and Metabolism* **67**, 1250-1259

Pell J.M., Saunders J.C. and Gilmour R.S. (1993) Differential regulation of transcription initiation from insulin-like growth factor-I (IGF-I) leader exons and tissue IGF-I expression in response to changed growth hormone and nutritional status in sheep. *Endocrinology* **132**, 1797-1807

Peter M.A., Winterhalter K.H., Boni-Schnetzler M., Froesch E.R. and Zapf J. (1993) Regulation of insulin-like growth factor-I (IGF-I) and IGF-binding proteins by growth hormone in rat white adipose tissue. *Endocrinology* **133**, 2624-2631

Philippe J. (1993) Somatostatin inhibits insulin - gene expression through a posttranscriptional mechanism in a hamster islet cell line. *Diabetes* **42**, 244-249

Philipps A.F., Peisson B., Hall K., Lake M., Skottner A., Sanengen T. and Sara V.R. (1988) The effects of biosynthetic insulin-like growth factor-I supplementation on somatic growth, maturation, and erythropoiesis on the neonatal rat. *Paediatric Research* **23**, 298-305

Phillips L.S. and Young H.S. (1976) Nutrition and somatomedin I. Effect of fasting and refeeding on serum somatomedin activity and cartilage growth activity in rats. *Endocrinology* **99**, 304-314

Plotsky P.M. and Vale W. (1985) Pattern of growth hormone releasing factor and somatostatin secretion into the hypophyseal-portal circulation of the rat. *Science (Wash. DC)* **230** 461-463

Pons S. and Torres-Aleman I. (1992) Basic fibroblast growth factor modulates insulin-like growth factor-I, its receptor, and its binding proteins in hypothalamic cell cultures. *Endocrinology* **131**, 2271-2278

Pørksen N., Munn S.R., Steers J.L., Veldhuis J.D. and Butler P.C. (1996) Effects of somatostatin on pulsatile insulin secretion: selective inhibition of insulin burst mass. *American Journal of Physiology, Endocrinolgy and Metabolism* **33**, E1043-E1049

Powell-Braxton L., Hollingsmead P., Warburton C., Dowd M., Pitts-Meck S., Dalton D., Gillett N. and Stewart T.A. (1993) IGF-I is required for normal embryonic growth in mice. *Genes Development* **7**, 2609-2617

Proudman J.A. and Wentworth B.C. (1978) Radioimmunoassay of turkey growth hormone. *General and Comparative Endocrinology* **36** 194-200

Rappolee D.A., Sturm K.S., Schultz G.A., Pedersen R.A. and Werb Z. (1989) The expression of growth factor ligands and receptors in preimplantation mouse embryos. In *Early embryo development and paracrine relationships*. Heyner S. and Wiley L. Editors

Read L.C., Lemmey A.B., Howarth G.S., Martin A.A., Tomas F.M. and Ballard F.J. (1991) The gastrointestinal tract is one of the most responsive target tissues for IGF-I and its potent analogs. In *Modern Concepts of Insulin-Like Growth Factors* pp 225-234 Ed. E.M. Spencer Elsevier Science Publishing Co. Inc., Amsterdam

Rechler M.M., Brown A.L. and Yang Y.W. (1991) Regulation of gene expression of rat insulin-like growth factor binding proteins 1 and 2. *Advances in Experimental Medicine and Biology* **293**, 137-148

Rechler M.M. (1993) Insulin-like growth factor binding proteins. In *Vitamins and Hormones* pp 1-14 Academic Press

Reinhardt R.R. and Bondy C.A. (1994) Insulin-like growth factors cross the blood-brain barrier. *Endocrinology* **135**, 1753-1761

Rennert N.J., Caprio S. and Sherwin R.S. (1993) Insulin-like growth factor I inhibits glucose-stimulated insulin secretion but does not impair glucose metabolism in normal humans. *Journal of Clinical Endocrinology and Metabolism* **76**, 804-806

Rinderknecht E. and Humbel R.E. (1976) Polypeptides with nonsuppressible insulin-like cell-growth-promoting activities in human serum: isolation, chemical characterization and some biological properties of forms I and II. *Proceedings of the National Academy of Science, USA* **73,** 2365-2369

Rinderknecht E. and Humbel R.E. (1978a) The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *Journal of Biological Chemistry* **253,** 2769-2776

Rinderknecht E. and Humbel R.E. (1978b) Primary structure of human insulin-like growth factor II. *FEBS Letters* **89,** 283-286

Ritvos O., Ranta T., Jalkanen J., Suikkari A.-M., Voutilainen R., Bohn H. and Rutanen E.M. (1988) Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. *Endocrinology* **122**, 2150-2157

Robbins R.J., Leidy Jr. J.W. and Landon R.M. (1985) The effects of growth hormone, prolactin, corticotropin and thyrotropin on the production and secretion of somatostatin by hypothalamic cells *in vitro. Endocrinology* **117**, 538-543

Roberts Jr. C.T., Brown A.L., Graham D.E., Seelig S., Berry S., Gabbay K.H. and Rechler M.M. (1986) Growth hormone regulates the abundance of insulin-like growth factor I RNA in adult rat liver. *Journal of Biological Chemistry* **261**, 10025

Rodriguez-Tarduchy G., Collins M.K., Garcia I. and Lopez Rivas A. (1992) Insulin-like growth factor-I inhibits apoptosis in IL-3-dependent hemopoietic cells. *Journal of Immunology* **149**, 535-540

Rogers K.V., Vician L., Steiner R.A. and Clifton D.K. (1988) The effects of hypophysectomy and growth hormone administration on preprosomatostatin messenger ribonucleic acid in the periventricular nucleus of the rat hypothalamus. *Endocrinolgy* **122**, 586-591

Rogers S.A. and Hammerman M.R. (1988) Insulin-like growth factor II stimulates production of inosotil triphosphate in proximal tubular basolateral membranes from canine kidney. *Proceedings of the National Academy of Sciences, USA* **85**, 4037-4041

Rogers S.A., Purchio A.F. and Hammerman M.R. (1990) Mannose-6-phosphate-containing peptides activate phospholipase C in proximal tubular basolateral membranes from canine kidney. *Journal of Biological Chemistry* **265**, 9722-9727

Rotwein P. (1986) Two insulin-like growth factor I messenger mRNAs are expressed in human liver. *Proceedings of the National Academy of Sciences, USA*, **83**, 77-81

Rotwein P.S., Pollock K., Didier D. and Krivi G. (1986) Organization and sequence of the human insulin-like growth factor I gene. *Journal of Biological Chemistry* **261,** 4828-4832

Rotwein P.S., Pollock K., Watson M. and Milbrandt J.D. (1987) Insulin-like growth factor gene expression during rat embryonic development. *Endocrinology* **121**, 2141-2144

Romanus J.A., Tseng L.Y-H., Yang Y.W-H. and Rechler M.M. (1989) The 34 kilodalton insulin-like growth factor binding proteins in human cerebrospinal fluid and the A673 rhabdomyosarcoma cell line are human homologues of the rat BRL-3A binding protein. *Biochemical and Biophysical Research Communications* **163**, 875-881

Salmon W.D. Jr. and Daughaday W.H. (1957) A hormonal controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *Journal of Laboratory and Clinical Medicine* **49,** 825-836

Sara V. and Hall K. (1990) Insulin-like growth factors and their binding proteins. *Physiological Reviews* **70**, 591-614

Sara V.R., Carlsson-Skwirut C., Andersson C., Hall K., Sjogren B., Holmgren A. and Jornvall H. (1986) Characterization of somatomedins from human fetal brain: identification of a variant from insulin-like growth factor I. *Proceedings of the National Academy of Sciences USA* **83**, 4904-4907

Sato M. and Frohman L.A. (1993) Differential effects of central and peripheral administration of growth hormone (GH) and insulin-like growth factor on hypothalamic GH-releasing hormone and somatostatin gene expression in GH-deficient dwarf rats. *Endocrinology* **133**, 793-799

Schwander J. and Mary J.L. (1993) The RIA for IGFBP-2 in man - a meagre catch? *Growth Regulation* **3**, 104-108

Schwander J., Kutoh E, Mary J-L. and Ritz L. (1994) The gene regulation of insulin-like growth factor binding protein-2 (IGFBP-2). In *The insulin-like growth factors and their regulatory proteins*. pp 151-161 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld Elsevier Science Publishing Co. Inc., Amsterdam

Schwartz J., Foster C.M. and Satin M.S. (1985) Growth hormone and insulin-like growth factors I and II produce distinct alterations in glucose metabolism in 3T3-F442A adipocytes. *Proceedings of the National Academy of Sciences, USA* **82**, 8724-8728

Seeburg P.H., Sias S., Adelman J., DeBoer H.A., Hayflick J., Jhurani P., Goeddel D.V. and Heyneker H.L. (1983) Efficient bacterial expression of bovine and porcine growth hormones. *DNA* **2**, 37-45

Sell C., Baserga R. and Rubin R. (1995) Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etopside-induced apoptosis. *Cancer Research* **55**, 303-306

Shemer J., Adamo M.L., Roberts C.T. Jr. and LeRoith D. (1992) Tissue-specific transcription start site usage in the leader exons of the rat IGF-I gene: evidence for the differential regulation in the developing kidney. *Endocrinology* **131**, 2793-2799

Shemer J., Adamo M., Wilson G.L., Heffez D., Zick Y. and LeRoith D. (1987) Insulin and insulin-like growth factor I stimulate a common endogenous phosphoprotein substrate (pp185) in intact neuroblastoma cells. *Journal of Biological Chemistry* **262**, 15476-15482

Shimasaki S., Koba A., Mercado M., Shimonaka M. and Ling N. (1989) Complementary DNA structure of the high molecular weight rat insulin-like growth factor binding protein (IGF-BP3) and tissue distribution of its mRNA. *Biochemical and Biophysical Research Communications* **165**, 907-912

Shimasaki S., Shimonaka M., Ui M., Inouye S., Shibata F. and Ling N. (1990) Structural characterization of follicle stimulating hormone action inhibitor in porcine ovarian follicular fluid. *The Journal of Biological Chemistry* **265**, 2198-2202

Shimasaki S., Tanahashi H., Onada N., Sumitomo S., Mickey G., Liu X.-J., Ling N., Li D. and Erickson G. (1994) Transcriptional and posttranscriptional regulation of IGFBP-4 and -5 in cultured rat granulosa cells. In *The insulin-like growth factors and their regulatory proteins*. pp 193-204 Eds. R.C. Baxter, P.D. Gluckman and R.G. Rosenfeld Elsevier Science Publishing Co. Inc., Amsterdam

Shimatsu A. and Rotwein P.S. (1987) Sequence of two rat insulin-like growth factor I mRNAs differing within the 5'-untranslated region. *Nucleic Acids Research* **15,** 7196

Shimizu M., Webster C., Morgan D.O., Blau H.M. and Roth R.A. (1986) Insulin and insulin-like growth factor receptors and responses in cultured human muscle cells. *American Journal of Physiology* **251,** E611-E615

Shuldiner A.R. Nirula A. Scott L.A. and Roth J. (1990) Evidence that *Xenopus Laevis* contain two different nonallelic insulin-like growth factor-I genes. *Biochemical and Biophysical Research Communications* **166,** 223-230

Silbergeld A., Mamet R., Laron Z. and Nevo Z. (1981) The effect of insulin-like growth factor (IGF) and of human serum on steps in proteoglycan synthesis. *Acta Endocrinologica* **97,** 503-507

Silverman B.L., Bettendorf M., Kaplan S.L., Brumbach M.M. and Miller W.L. (1989) Regulation of growth hormone (GH) secretion by GH-releasing factor, somatostatin, and insulin-like growth factor I in ovine fetal and neonatal pituitary cells *in vitro*. *Endocrinology* **124**, 84-89

Simmons J.G., van Wyk J.J., Hoyt E.C. and Lund P.K. (1993) Multiple transcription start sites in the rat insulin-like growth factor-I gene give rise to IGF-I mRNAs that encode different IGF-I precursors and are processed differently *in vitro*. *Growth Factors* **9**, 205-221

Skottner A., Clark R.G., Fryklund L. and Robinson I.C. (1989) Growth responses in a mutant dwarf rat to growth hormone and recombinant human insulin-like growth factor I. *Endocrinology* **124**, 2519-2526

Smith V.G., Leman A.D., Seaman W.J. and VanRavenswaay F. (1991) Pig weaning weight and changes in hematology and blood chemistry of sows injected with recombinant porcine somatotropin during lactation. *Journal of Animal Science* **69**,3501-3510

Spencer G.S.G., Aitken W.M., Hodgkinson S.C. and Bass J.J. (1992) Neuroendocrine regulation of growth hormone secretion in sheep. v. growth hormone releasing factor and thyrotropin releasing hormone. *Domestic Animal Endocrinolgy* **9**, 115-123

Steeb C.B., Trahair J.F., Tomas F.M. and Read L.C. (1994) Prolonged administration of IGF peptides enhances growth of gastrointestinal tissues in normal rats. *American Journal of Physiology* **266**, G1090-G1098

Steele-Perkins G., Turner J., Edman J.C., Hari J., Pierce S.B., Stover C., Rutter W.J. and Roth R.A. (1988) Expression and characterization of a functional human insulin-like growth factor receptor. *Journal of Biological Chemistry* **263**, 11486-11492

Strain A.J., Hill D.J., Swenne L. and Milner R.D.G. (1987) The regulation of DNA synthesis in human fetal hepatocytes by placental lactogen, growth hormone and insulin-like growth factor I/somatomedin-C. *Journal of Cell Physiology* **132**, 33-40

Straus D.S., Ooi G.T., Orlowski C.C. and Rechler M.M. (1991) Expression of the genes for insulin-like growth factor I (IGF-I), IGF-II and IGF-binding proteins-1 and -2 in fetal rat under conditions of intauterine growth retardation caused by maternal fasting. *Endocrinology* **128**, 518-525

Suikkari A.M., Jalkanen J., Koistinen R., Butzow R., Ritvos O., Ranta T. and Seppala M. (1989) Human granulosa cells synthesize low molecular weight insulin-like growth factor-binding protein. *Endocrinolgy* **124**, 1088-1090

Tannenbaum G.S. (1983) Insulin-like growth factors: A role in growth hormone negative feedback and body weight regulation via brain. *Science* **220** 77-79

Tannenbaum G.S. (1989) Interrelationship of growth hormone releasing hormone and somatostatin in the regulation of growth hormone secretion. In *Hormonal Regulation of Growth*. H. Freich and M.O. Thorner (Ed.) Raven Press, New York. 1-17

Tavakkol A., Simmen F.A. and Simmen R.C.M. (1988) Porcine insulin-like growth factor-I (pIGF-I): Complementary deoxyribonucleic acid cloning and uterine expression of messenger ribonucleic acid encoding evolutionarily conserved IGF-I peptides. *Molecular Endocrinology* **2**, 674-681

Termanini B., Nardi R.V., Finan T.M., Parikh I. and Korman L.Y. (1990) Insulin-like growth factor I receptors in rabbit gastrointestinal tract: Characterisation and autoradiographic localization. *Gastroenterology* **99**, 51-60

Thomas M.J., Kikuchi K., Bichell D.P. and Rotwein P. (1994) Rapid activation of rat insulin-like growth factor-I gene transcription by growth hormone reveals no alterations in deoxyribonucleic acid-protein interactions within the major promoter. *Endocrinology* **135**, 1584-1592

Thomas M.R., Miell J.P., Taylor A.M., Ross R.J.M., Arnao J.R., Jewitt D.E. and McGregor A.M. (1993) Endocrine and cardiac paracrine actions of insulin-like growth factor-I (IGF-I) during thyroid disfunction in the rat: is IGF-I implicated in the mechanism of heart/body weight change during abnormal thyroid function? *Journal of Molecular Endocrinology* **10**, 313-323

Thompson J.L., Butterfield G.E., Marcus R., Hintz R.L., Van Loan M., Ghiron L. and Hoffman A.R. (1995) The effects of recombinant human insulin-like growth factor-I and growth hormone on body composition in elderly women. *Journal of Clinical Endocrinology and Metabolism* **80**, 1845-1852

Tomas F.M., Campbell R.G., King R.H., Johnson R.J., Chandler C.S. and Taverner M.R. (1992a) Growth hormone increases whole-body protein turnover in growing pigs. *Journal of Animal Science* **70**, 3138-3143

Tomas F.M. Knowles S.E., Chandler C.S., Francis G.L., Owens P.C. and Ballard F.J. (1993) Anabolic effects of insulin-like growth factor-I (IGF-I) and an IGF-I variant in normal female rats. *Journal of Endocrinology* **137**, 413-421

Tomas F.M., Knowles S.E., Owens P.C., Read L.C., Chandler C.S., Gargosky S.E. and Ballard F.J. (1991a) Effects of full length and truncated insulin-like growth factor-I on nitrogen balance and muscle protein metabolism in nitrogen-restricted rats. *Journal of Endocrinology* **128**, 97-105

Tomas F.M., Knowles S.E., Owens P.C., Read L.C., Chandler C.S., Gargosky S.E. and Ballard F.J. (1991b) Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with insulin-like growth factor (IGF)-I and des (1-3) IGF-I. *Biochemical Journal* **276**, 547-554

Tomas F.M. Knowles S.E., Owens P.C., Chandler C.S., Francis G.L., Read L.C. and Ballard F.J. (1992b) Insulin-like growth factor-I (IGF-I) and especially IGF-I variants are anabolic in dexamethasone treated rats. *Biochemical Journal* **282**, 91-97

Tricoli .V., Rall L.B., Scott J., Bell G.I. and Shows T.B. (1984) Localization of insulin-like growth factor genes to chromosomes 11 and 12. *Nature* **310**, 784-786.

Tseng L.Y-H., Ooi G.T., Brown A.L., Staus D.S. and Rechler M.M. (1992) Transcription of the insulin-like growth factor binding protein-2 gene and identification of a functional promoter lacking a TATA box. *Molecular Endocrinology* **4**, 2039-2051

Tuggle C.K. and Trenkle A. (1996) Control of growth hormone synthesis. *Domestic Animal Endocrinology* **13**, 1-33

Turkalj I., Keller U., Ninnis R., Vosmeer S. and Stauffacher W. (1992) Effect of increasing doses of recombinant human insulin-like growth factor-I on glucose, lipid, and leucine metabolism in man. *Journal of Clinical Endocrinology and Metabolism* **75**, 1186-1191

Uchiyama T., Kaji H., Abe H. and Chihara K. (1994) Negative regulation of hypothalamic growth hormone releasing factor messenger ribonucleic acid by growth hormone and insulin-like growth factor I. *Neuroendocrinology* **59**, 441-450

Uemasu J., Fujihara M. and Kawasaki H. (1996) Effects of low dose of recombinant human growth hormone on renal excretion of insulin-like growth factor-I (IGF-I) in humans: an inverse relation between serum and urinary IGF-I level. *Clinical Nephrology* **46**, 1-5

Ullrich A., Gray A., Tam A.W. *et al.* (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO Journal* **5**, 2503-2512

Underwood L.E., D'Ercole A.J. Clemmons D.R. and Van Wyk J.J. (1986) Paracrine functions of somatomedin. In *Clinics in Endocrinology and Metabolism: Paracrine control,* **15,** 59-77, Ed. P. Franchimont. London: W.B. Saunders Co.

Unterman T.G., Simmons R.A., Glick R.P. and Ogata E.S. (1993) Circulating levels of insulin, insulin-like growth factor-I (IGF-I), IGF-II and IGF-binding proteins in the small for gestational age fetal rat. *Endocrinology* **132**, 327-336

van Buul-Offers S., Ueda I. and Van den Brande J.L. (1986) Biosynthetic somatomedin C (SM-C/IGF-I) increases the length and weight of Snell dwarf mice. *Pediatric Research* **20**, 825-827

VandeHaar M.J., Moats-Staats B.M., Davenport M.L. Walker J.L. Ketelslegers J.M., Sharma B.K. and Underwood L.E. (1991) Reduced serum concentrations of insulin-like growth factor-I (IGF-I) in protein-restricted growing rats are accompanied by reduced IGF-I mRNA levels in liver and skeletal muscle. *Journal of Endocrinology* **130**, 305-312

Veomett G.E., Munger L.L., Smith G.L. and Schollmeyer J.E. (1989) Heterogeneity of insulin-like growth factor binding proteins in swine. *Molecular and Cellular Endocrinology* **65**, 49-57

Verspohl E.J., Roth R.A., Vigneri R. and Goldfine I.D. (1984) Dual regulation of glycogen metabolism by insulin and insulin-like growth factors in human hepatoma cells (Hep-G2): analysis with an anti-receptor monoclonal antibody. *Journal of Clinical Investigation* **74**, 1436-1443

Vetter U., Zapf J., Heit W., Helbing G., Heinze E., Froesch E.R. and Teller W.M. (1986) Human fetal and adult chondrocytes. Effects of insulin-like growth factors I and II, insulin and growth hormone on clonal growth. *Journal of Clinical Investigation* **77**, 1903-1908

Walton P.E. and Etherton T.D. (1989) Effects of porcine growth hormone and insulin-like growth factor-I (IGF-I) on immunoreactive IGF-binding protein concentrations in pigs. Journal of *Endocrinology 120*, 153-160

Walton P.E., Etherton T.D. and Chung C.S. (1987) Exogenous pituitary and recombinant growth hormones induce insulin and insulin-like growth factor I resistance in pig adipose tissue. *Domestic Animal Endocrinology* **4**, 183- 189

Walton P.E., Gopinath R., Burleigh B.D. and Etherton T.D. (1989b) Administration of recombinant human insulin-like growth factor I to pigs: Determination of circulating half-lives and chromatographic profiles. *Hormone Research* **31**, 138-142

Walton P.E., Gopinath R. and Etherton T.D. (1989a) Porcine insulin-like growth factor (IGF) binding protein blocks IGF-I action on porcine adipose tissue. *Proceedings of the Society for Experimental Biology and Medicine* **190**, 315-319

Walton P.E., Owens P.C., Knowles S.E., Dunaiski V., Grant P.A., Tomas F.M. and Dunshea F.R. (1994) Effects of IGF-I analogues on growth and metabolism in pigs. *Growth Regulation* **4** Suppl.1, 86

Walton, P.E., Tomas F.M., Owens P.C., Priebe I.K., Burgoyne J.L., Francis G.L. and Dunshea F.R. (1993) Effects of potent insulin-like growth factor I analogues on plasma glucose and amino acids in pigs. *Manipulating Pig Production* **5**, 185

Ward A. and Elliss C.J. (1992) The insulin-like growth factor genes. In *The insulin-like growth factors: structure and biological functions*. Schofield P.N. Editor pp 45-79

Wardzala L.J., Simpson I.A., Rechler M.M. and Cushman S.W. (1984) Potential mechanism of the stimulatory action of insulin on insulin-like growth factor II binding to the isolated rat adipose cell: apparent redistribution of receptors cycling between a large intracellular pool and the plasma membrane. *Journal of Biological Chemistry* **259**, 8378-8383

Weber M.M., Melmed S., Rosenbloom J., Yamasaki H. and Prager D. (1992) Insulin-like growth factor 2 action in rat somatotrophs is mediated by the insulin-like growth factor I receptor. *Endocrinolgy* **131**, 2147-2153

Weeden T.L., Nelssen J.L., Goodband R.D., Hansen J.A., Fitzner G.E., Friesen K.G. and Laurin J.L. (1993) Effects of porcine somatotropin and dietary phosphorous on growth performance and bone properties of gilts. *Journal of Animal Science* **71**, 2674-2682

Weller P.A., Dauncey M.J., Bates P.C., Bramfeld J.M., Buttery P.J. and Gilmour R.S. (1994) Regulation of porcine insulin-like growth factor I and growth hormone receptor mRNA expression by energy status. *American Journal of Physiology* **266**, E776-E785

Weller P.A., Dickson M.C., Huskisson N.S. Dauncey M.J., Buttery P.J. and Gilmour R.S. (1993) The porcine insulin-like growth factor-I gene: characterization and expression of alternate transcription sites. *Journal of Molecular Endocrinology* **11**, 201-211

Williams E.D. (1988) The pathobiology of thyroid tumours. *J. Endocrinology* **117**(Suppl.) Abstract 5

Wolverton C.K., Azain M.J., Duffy J.Y., White M.E. and Ramsay T.G. (1992) Influence of somatotropin on lipid metabolism and IGF gene expression in porcine adipose tissue. *American Journal of Physiology* **263**, E637-E645

Woychik R.P., Camper S.A., Lyons R.H., Horowitz S., Goodwin E.C. and Rottman F.M. (1982) Cloning and nucleotide sequencing of the bovine growth hormone gene. *Nucleic Acid Research* **10**, 7197-7210

Wray-Cahen D., Boyd R.D., Bauman D.E. and Ross D.A. (1993) Effect of porcine somatotropin on the response of growing pigs to acute challenges of glucose, insulin and epinephrine and during a hyperinsulinemic-euglycemic clamp. *Domestic Animal Endocrinology* **10**, 103-115

Xue Z.G., Le Douarin N.M. and Smith J. (1988) Insulin and insulin-like growth factor- can trigger the differentiation of catecholaminergic precursors in cultures of dorsal root ganglia. *Cell Differentiation and Development* **25**, 1-10

Xu R-J., Mellor D.J., Birtles M.J., Breier B.H. and Gluckman P.D. (1994) Effects of oral IGF-I or IGF-II on digestive organ growth in newborn piglets. *Biology of the Neonate* **66**, 280-287

Yamashita S. and Melmed S. (1986a) Insulin regulation of rat growth hormone gene transcription. *Journal of Clinical Investigation* **48**, 1008-1014

Yamashita S. and Melmed S. (1986b) Insulin-like growth factor I action on rat anterior pituitary cells: suppression of growth hormone secretion and messenger ribonucleic acid levels. *Endocrinology* **118**, 176-182

Yang Y.W.-H., Robbins A.P., Nissley S.P. and Rechler M.M. (1991) The chick embryo fibroblast cation-independent mannose 6-phosphate receptor is functional and immunologically related to the mammalian insulin-like growth factor-II (IGF-II)/Man 6-P receptor but does not bind IGF-II. *Endocrinology* **128**, 1177-1189

Young G.P., Taranto T.M., Jonas H.A., Cox A.J., Hogg A. and Werther G.A. (1990) Insulin-like growth factors and the developing and mature rat small intestine: receptors and biological actions. *Digestion*, **46** (suppl 2) 240-252

Zapf J., Hauri C., Waldvogel M. and Froesch E.R. (1986) Acute metabolic effects and half-lives of intravenously administered insulin-like growth factors I and II in normal and hypophysectomized rats. *Journal of Clinical Investigation* **77**, 1768-1775

Zapf J., Hauri C., Waldvogel M., Futo E., Hasler H., Binz K., Guler H.P., Schmid C. and Froesch E.R. (1989) Recombinant insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. *Proceedings of the National Academy of Animal Science USA* **86**, 3813-3817

Zapf J., Schmid C., Guler H.P. *et al.* (1990) Regulation of binding proteins for insulin-like growths (IGF) in humans. Increased expression of IGF binding protein 2 during IGF treatment of healthy adults and in patients with extrapancreatic tumour hypoglycaemia. *Journal of Clinical Investigations* **86**, 952-961

Zeytin F.N., Rusk S.F. and De Lellis R. (1988) Growth hormone-releasing factor and fibroblast growth factor regulate somatostatin gene expression. *Endocrinology* **122**, 1133-1139

Zhao X. and Donovan S.M. (1995) Combined growth hormone (GH) and insulin-like growth factor-I (IGF-I treatment is more effective than GH or IGF-I alone at enhancing recovery from neonatal malnutrition in rats. *Journal of Nutrition* **125**, 2773-2786

Zick Y., Sasaki N., Rees-Jones R.W., Grunberger G., Nissley P.S. and Rechler M.W. (1984) Insulin-like growth factor-I (IGF-I) stimulates tyrosine kinase activity in purified receptors from a rat liver cell line. *Biochemical and Biological Research Communications* **119**, 6-13

Zing A.E. and Froesch E.R. (1973) Effects of partially purified preparations with nonsuppressible insulin-like activity (NSILAs) on sulfate incorporation into rat and chicken cartilage. *Diabetology* **9,** 472-47