GROWTH HORMONE (GH) AND INSULIN-LIKE GROWTH FACTOR-I (IGF-I) *IN VIVO* : INVESTIGATION VIA TRANSGENESIS IN RATS

by

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Summary

With the eventual aim of producing a transgenic rat model in which to assess the ability of IGF-I to inhibit muscle degradation during stress, I have constructed a chimeric transgene linking the promoter of the Phosphoenolpyruvate Carboxykinase (PEPCK) gene to an IGF-I cDNA. This construct was permanently transfected into Chinese Hamster Ovary (CHO) cells and was shown to enable production of biologically active hIGF-I by these cells. Similar transfections of the construct into rat H4IIE hepatoma cells did not result in the production of significant biologically active hIGF-I. This is likely to result as a consequence of a feedback loop in which hIGF-I is able to inhibit transcription via the PEPCK promoter in the transfected cells.

Prior to the use of the PEPCK-IGF-I construct for transgenesis, the rat system was evaluated using a transgene construct proven previously in mice, hMTIIa-pGH. Rats carrying the MTIIa-pGH transgene were successfully generated at a frequency comparable to mice. Founder rats produced pGH in their blood at the time of weaning at concentrations between 18ng/ml -1200 ng/ml. While most animals with plasma GH levels of >25 ng/ml at weaning exhibited enhanced growth relative to their littermates, other animals exhibited apparently normal growth in the presence of pGH. Examination of the tissue-site of pGH expression in several of the primary transgenic rats showed that in almost all cases the liver was the major producer of pGH mRNA. In one animal, the intestine appeared to be the major producer of pGH mRNA. Two families of rats were generated from breedings. In both families the production of pGH and associated growth phenotype was co-inherited with the transgene. In one of the families three of the transgenic F1 progeny exhibited plasma levels of pGH which were inducible by zinc treatment.

The PEPCK-IGF-I transgene was subsequenly microinjected into rat oocytes and transgenic animals generated with a similar frequency to the MTIIa-pGH transgene. Expression of hIGF-I was detected in 1/11 rats into which the transgene had integrated. This animal produced approximately 30 ng/ml of hIGF-I following fasting. Analysis of hIGF-I mRNA distribution showed that expression was predominant in the liver and kidney of the animal. This animal produced an F1 generation, 50 % of which were shown to inherit the transgene in the same organisation as their transgenic parent. Blood was sampled from these animals after fasting for 24 hours and following re-feeding. Analysis

of these samples by RIA showed that 3/6 of the transgene positive animals were expressing hIGF-I. Two of these animals exhibited expression which was induced by the fasting and subsequently depressed by re-feeding. The third animal showed essentially constant levels of hIGF-I. Expression of hIGF-I mRNA has been analysed in these animals and in all cases the liver was the predominant expression site with significant levels also being observed in the kidneys. The PEPCK-IGF-I transgene has been faithfully inherited into the F2 generation of this line and expression of hIGF-I mRNA has also been observed in the livers and kidneys of transgenic F2 generation animals. No effects on the growth rate of transgenic animals in this litter have been observed probably due to the extremely low basal hIGF- levels of expression likely in these animals.

Statement

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

Nicholas Kallincos

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Publications Arising From Research

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

1.1 Growth Regulation

Regulation of growth in vertebrate organisms is governed by several different hormones and peptide growth factors produced by different tissues at specific developmental timepoints. The growth response is manifested both as a result of endocrine release of these factors and paracrine/autocrine release and actions. Several of the key hormones involved in the phenomena of growth in vertebrates have been identified and characterised extensively.

The central control of mammalian growth is believed to be via the growth hormone (GH) cascade. This series of events begins in the hypothalamus, where neurotransmitters stimulate the release of growth hormone releasing factor (GHRF). This small peptide is transported to the pituitary where it induces the secretion of GH from somatotroph cells, into the bloodstream. Alternatively somatostatin (SRIF) can inhibit release of GH from the anterior pituitary. GH is transported through the circulatory system to the liver where it stimulates the production of insulin-like growth factor-I (IGF-I). Many, if not all of growth hormone's growth-promoting actions are believed to be due to this production of IGF-I (Bang et al., 1993). This idea formed the basis for the "somatomedin hypothesis" proposed by Salmon and Daughaday in 1957. Additionally, IGF-I is able to inhibit pituitary GH synthesis, providing a necessary feedback control of this axis (Berelowitz et al., 1981; Yamashita et al., 1987). Hence both GH and the insulin-like growth factors play a pivotal role in the phenomena of growth. A detailed discussion of the role played by each of these molecules will be presented in this chapter.

In addition, to these hormones/growth factors, other molecules are involved in the regulation of mammalian growth eg. insulin, somatostatin, thyroid hormone, androgens, estrogens and corticosteroids. These will only be discussed where they impinge upon the GH/IGF-I axis. Discussions will be focussed in particular on the role of the GH/IGF-I axis in the growth of rodents.

1.2 Growth Hormone

GH is a polypeptide member of a family of related proteins which includes prolactin (PRL) and in humans, placental lactogen (PL) (Watahiki et al., 1989; Nicoll et al., 1986). This protein is secreted by the somatotroph cells of the anterior pituitary in an episodic

pulsatile manner in every mammalian species in which it has been examined (Martin et al., 1978).

A link between this pituitary derived product and growth regulation was first suspected in acromegalic humans that contained enlarged pituitaries (Marie, 1889). Subsequently, the importance of the pituitary in other forms of human gigantism and dwarfism was noted. The direct role of the pituitary was shown by the observation that hypophysectomy resulted in the immediate cessation of growth in young dogs and rats (Smith, 1927; Eigenmann et al., 1977).

Li et al (1945) were the first to isolate GH from bovine pituitaries. Since that time it has been isolated from human pituitaries and with the advent of recombinant DNA technology, produced biosynthetically on a large scale. Administration of GH to hypophysectomised rats has been shown to increase growth (Russel et al., 1985; Schoenle et al., 1985; Guler et al., 1988), while ablation of the pituitary somatotroph cells in transgenic mice has been shown to result in dwarf animals (Borreli et al., 1989) reinforcing the importance of this hormone in animal growth. Furthermore, hGH has been used for over 30 years in the treatment of children with GH insufficiency and consequent growth defects (Raben et al., 1958).

Growth hormone has both direct and indirect (via the IGFs) effects. It exhibts wide ranging short term metabolic effects including increasing protein synthesis and amino acid uptake by cells, effects on carbohydrate and lipid metabolism (on the enzymes of glycogen synthesis / degradation and of gluconeogenesis) and both insulin-like (direct and mediated by the IGFs) and insulin-antagonistic effects (see review by Davidson, 1987). The role of these short-term effects in somatic growth is not fully understood. In contrast to the short-term effects, good evidence supports the hypothesis that many (if not all) of the longer term growth effects are due to IGF-I release (both from liver and other tissues) in response to GH (Schlechter et al., 1986; Isgaard et al., 1988).

GH is a polypeptide of 188-191 amino acids depending on the species, containing four cysteine residues which form two disulphide bridges. The position of the four cysteine residues is conserved amongst all GHs, prolactins (PRLs) and placental lactogens (PLs), indicating the structural/functional importance of these residues (Watahiki et al., 1989).

GH is synthesised by the somatotroph cells of the anterior pituitary in a pro-hormone form containing a hydrophobic leader sequence (between 22-26 amino acids) which is cleaved from the molecule during secretion (Seeburg et al., 1977). In addition to the

normally secreted 22 kDa form, a number of GH s which differ in molecular weight have been detected. Thus, the pituitary releases a number of GH forms into the circulation with the major species being a 22 kDa form. These alternative GHs arise from alternative splicing events, aggregation of native GH, inter-chain disulphide linkages, glycosylation differences, phosphorylation and proteolytic modifications of the molecule (for review see Baumann, 1991; Lewis , 1992.).

The most notable human variant is 20 kDa in size and results from an alternative splicing event in exon 3 of the human GH gene that deletes amino acids 32-46 of the polypeptide (Wallis et al., 1980). Such a variant has also been detected in the rat and is likely to be present in all species (Sinha et al., 1986; Howland et al., 1987). Importantly, the biological properties of this variant molecule are somewhat different to the normally secreted 22 kDa form. This molecule does not bind to the GH receptors in human liver and as result is unable to stimulate significant production of IGF-I from this organ (McCarter et al., 1990). The 20 kDa variant species lacks the insulin-like properties of the 22 kDa form. Hence, GH may have as yet undetermined functions by virtue of these structural variants.

The genes encoding rat, human, bovine, porcine and ovine GH have been isolated and each contains five exons and four introns of similar lengths in almost identical positions (Barta et al ., 1981; DeNoto et al., 1981; Seeburg et al., 1977; Vize et al., 1987; Woychick et al., 1982; Byrne et al., 1987). Furthermore, these genes exhibit striking sequence conservation in both their 5'-promoter and 3'-untranslated regions (Woychick et al., 1982; Vize et al., 1987). The similar arrangement and high degree of homology is consistent with the suggestion that these genes have a common evolutionary origin.

Although, single copy GH genes are present in the rat, bovine, porcine and ovine genomes, there are five GH related genes in the human haploid genome (Seeburg et al., 1982) Three of these genes encode placental lactogen (hPL) while two encode hGH. Only one of the hGH genes termed hGH-N is expressed in the pituitary. A second gene (hGH-V) is expressed in the placenta (Frankenne et al., 1987) and encodes a variant hGH which differs from the normal protein product at 13 positions (Lewis et al., 1992). Only the pituitary expressed hGH-N gene is capable of generating the 20 kDa variant protein discussed previously (Baumann et al., 1991).

Analysis of GH gene transcription *in vitro* in pituitary cell lines and *in vivo*, has shown the GH gene to be stimulated by growth hormone releasing factor (GHRF) (Baringa et al., 1983), thyroid hormone (Dobner et al. ,1981; Evans et al., 1982) and glucocorticoids

(Baringa et al., 1983; Spindler et al., 1982). While all of these agents act to increase initiation of transcription, thyroid hormone and glucocorticoids are believed to also act at a post-transcriptional level. In addition to these stimulatory agents, somatostatin and IGF-I are able to inhibit the secretion of GH (Yamashita et al., 1986). IGF-I's inhibitory action also involves attenuation of GH transcription and requires binding to type 1 IGF receptor present on somatotrophs (Yamashita et al., 1987; Yamasaki et al., 1991).

Significant progress has been made in identifying regulatory sequences and protein trans-acting factors responsible for the regulation of the GH genes. There is extensive homology within the first 500 bp of the 5' flanking regions of the GH genes from rat and human. The first regions of homology correspond to the TATA box and binding sites for GHF-1 (or Pit -1), the pituitary-specific transcription factor (Bodner et al., 1988; Ingraham et al., 1988). This factor has been isolated an shown to be a 291 amino acid protein containing a 60 amino acid homeodomain and a 75 amino acid POU type domain (Bodner et al., 1988; Ingraham et al., 1988). Like other transcriptional activators these two binding domains map to the C-terminal part of the molecule while the N-terminus contains a strongly negatively charged activation domain (Karin et al., 1990). It has recently been shown that although both GHF-I elements in the rat gene are required for pituitary specific expression, maximal levels of GH expression require a synergistic interaction with upstream promoter elements (Lira et al., 1993).

GHRF induces transcription of both the rat and human GH genes via a mechanism involving cAMP (Baringa et al., 1985). Thus, a third region of homology, responsible for this induction by cAMP has been mapped between the TATA box and position -100 (Dana et al., 1989; Copp et al., 1989; Thomas et al., 1990).

Two further elements in the 5' flanking region mediate induction of the human gene by glucocorticoids. In cooperation with an element within the first intron of this gene these sequences enable binding of the glucocorticoid receptor (Slater et al., 1985). Furthermore, a thyroid response element has been mapped in the 5' DNA of the rat GH gene (Wight et al., 1988; Glass et al., 1988). More recently a series of silencer elements have been identified in the promoter of the rat growth hormone gene (Roy et al 1992). It is believed that binding of a protein factor to these sites represses growth hormone expression in non-pituitary cells.

1.3 GH Receptors

The onset of GH regulation of mammalian somatic growth occurs postnatally and appears to be correlated with the appearance of specific GH receptors (Gluckman et al., 1983; Freemark et al., 1986) The initial step in the action of GH is its binding to a cell surface receptor. As with the hormone itself, the receptor for growth hormone is part of a gene family (Boutin et al., 1988) which contains the receptors for GH, Prolactin (PRL) and several cytokines (Bazan et al., 1989)(see Kelly et al., 1993 for an extensive review of this family). In addition, a soluble binding protein for GH has been identified in a number of species. Together these molecules are responsible for mediating and modulating the range of biological actions of GH.

Although, the highest concentrations of this receptor (and its mRNA) are found in the mammalian liver, the GH receptor has now been shown to be present in a wide range of mammalian tissues (adipose, intestine, kidney, heart, lung, pancreas, brain, cartilage, skeletal muscle, testes and immune cells) (Kelly et al., 1991), suggesting that GH has direct actions in these tissues. Consistent with the heterogeneity of GH, marked heterogeneity in GH receptors has been shown (Barnard et al., 1985; Smith et al., 1987) and it has been suggested that there are at least three subtypes of these receptors (Goodowski et al., 1989).

The rabbit liver GH receptor was the first to be purified and cloned (Leung et al., 1987). The primary structure of the mature receptor as deduced from the nucleotide sequence resulted in a 620 amino acid protein containing an extracellular hormone-binding domain of 246 amino acids (seven cysteine residues and 5 potential glycosylation sites), a single transmembrane spanning region and a cytoplamic domain of 350 amino acids. Subsequently, the mouse, rat, bovine and ovine receptors have been cloned and all share approximately 70 % amino acid similarity (Mathews et al., 1989; Hauser et al., 1990; Adams et al., 1990).

Investigations on the regulation of the GH receptor have revealed that its levels are decreased in situations of growth failure in rats e.g., fasting, diabetes and renal failure (Postel-Vinay et al., 1982; Finidori et al., 1980). Receptor levels respond variably to GH, in some instances being up-regulated eg. expression of ovine GH in transgenic mice (Orian et al., 1991) and in others e.g. hypophysectomy or diabetes in female rats, down-regulation of receptors is evident (Kelly et al., 1991). In these cases the levels of GH mRNA do not change, suggesting that this regulation occurs post-transcriptionally. The observation that insulin treatment of streptozotocin diabetic rats can restore hepatic GH receptors suggests

that insulin also plays a role in their regulation (Baxter et al., 1980; Postel-Vinay et al., 1982). Additionally, estrogen appears to modulate the levels of the receptor as shown by the 10-fold increase in GH binding to liver membranes from pregnant rats (Kelly et al., 1991).

GH has been shown to have effects on the growth of chondrocyte and osteoblast cultures, stimulation of local IGF-I production has also been shown to result from these treatments suggesting that both hormones may act coordinately on cells (Madsen et al., 1983; Stracke et al. ,1984). These observations have been strengthened by *in vivo* studies in which concurrent administration of GH and IGF-I antibodies to hypophysectomised rats was shown to abolish the stimulation of tibial growth normally observed with GH alone (Schlechter et al., 1986). As well as regulating the production of IGF-I from a variety of cells in culture (liver and others) (Mathews et al., 1986), GH has been shown to have a direct effect on pre-adipocyte differentiation (Green et al., 1985).

Despite the characterisation of the GH receptors at a molecular biological and structural level, lack of suitable *in vitro* models for direct GH action (independent of IGF-I production) has made the study of the signal transduction pathway difficult and to date the mechanism by which GH effects biological responses after binding to the receptor is still unresolved. A more recent addition to this area of research, was the suggestion that receptor dimerisation was probably crucial for signal transduction. This suggestion followed the determination of a crystal structure for the hGH/GH receptor extracellular domain complex (DeVos et al., 1992).

1.4 GH Binding Proteins

While circumstantial evidence for the presence of a soluble serum GHBPs existed for many years, by virtue of high molecular weight forms of circulating GH, it was not until recently that their existence has been substantiated. Two groups of researchers almost simultaneously discovered the high affinity GH-BP. A lower affinity GH-BP has been isolated (Baumann et al., 1990) but its relevance to growth hormone function is at this stage debatable. Baumann et al. (1986) discovered the high affinity GH-BP in examining the higher molecular weight forms of GH found in plasma and their conversion to smaller forms. At the same time Herington et al., (1986) identified a soluble GH receptor-like protein in rabbit liver cytosol and by extension in rabbit and human serum. High affinity GHBPs, homologous to that present in human plasma have now been identified in the

rabbit, the pig, the pregnant mouse and the rat (Ymer et al., 1985; Leung et al., 1987; Spencer et al. ,1988; Smith et al., 1989; Shaw et al., 1988; Lauterio et al., 1988).

The high affinity GHBPs are glycosylated proteins composed of approximately 620 amino acids. These 60 kDa proteins bind one molecule of GH each to form larger complexes (Baumann et al., 1991). It is now widely accepted, but not definitively shown for all species, that these proteins are essentially the extracellular domains of the GH receptor.

Molecular biology has provided great information into the mechanism of generation and regulation of these GHBPs, particularly in the rat. Following the isolation of a liver cDNA for the rat GH receptor (Mathews et al., 1989), an alternatively spliced truncated form of the mRNA was identified in both mouse and rat (Smith et al., 1989; Baumbach et al., 1989) suggesting a convenient mechanism for generation of the BP. Generation of the soluble GHBP by alternative splicing has not been shown for humans and proteolytic modification of the GH receptor cannot yet be ruled out .

In the rat, the soluble GHBP is encoded in a 1.2 kb mRNA species while the GH receptor is represented by a 4.5 kb transcript (Tiong et al., 1991,1992). In addition to these species another transcript of 2.6 kb which contains GHBP specific sequences has also been detected in rat liver (Carlsson et al. ,1990; Tiong et al 1991). The significance of these additional transcripts is unknown but their importance is supported by their presence in other species and non-hepatic tissues (Herington et al., 1991). Studies on the major GHBP mRNA have shown that its major site of expression is the liver with lower levels present in other tissues expressing the GH receptor. Also the GHBP exhibits a similar ontological expression in the liver to the GH receptor (Tiong et al., 1992), with low expression in the fetus and increasing expression postnatally. Differences in the postnatal expression of the BP mRNA and the receptor mRNA suggest that they are each regulated independently (Herington et al., 1991). This is supported by the large increase in GHBP but not GH receptor mRNA during pregnancy in the rat (Herington et al., 1991).

With the finding that the receptor and binding protein for GH are regulated differently, it is likely that the binding protein plays a specific role in the modulation of growth hormone action. This role has yet to be elucidated but may involve preventing fluctuations in the amount of GH available to target tissues, which would otherwise occur due to the pulsatile nature of GH secretion (Herington et al., 1991). Also the GH binding protein may act as a store of GH, increasing its serum half life (Herington et al., 1991;

Baumann et al., 1987). Furthermore, it is possible that the binding protein may modulate the action of GH (enhance or inhibit), as has been shown for the IGFBPs (see later). In this regard it is interesting that the mRNA for the binding protein has been identified in many other tissues apart from the liver, eg. skin (Lobie et al., 1989; Garcia-Aragon et al., 1992) implying that it may control actions of GH in as yet undefined target tissues for GH action.

1.5 The Insulin-like Growth Factors

1.5.1 Historical aspects

The insulin-like growth factors are a class of small molecular weight polypeptides which by virtue of their structural similarities to insulin are able to mimic some of the biological effects of insulin. The insulin-like growth factors in addition exhibit pronounced effects on cellular growth, unobtainable with insulin. On the basis of their receptor binding, immunological and structural characteristics, two subclasses of insulin-like growth factors have been distinguished; Insulin-like growth factor -I (IGF-I) and Insulin-like growth factor-II (IGF-II). I will deal almost exclusively with IGF-I which is of major importance in postnatal mammalian growth, although where relevant, discussions of IGF-II will also be included.

The study of the insulin-like growth factors originally resulted from three independent lines of observation. In 1958 Salmon and Daughaday observed that serum from growth hormone treated hypophysectomised rats stimulated the incorporation of sulphate into cartilage *in vitro* whereas growth hormone itself was without effect in this assay. These observations prompted the suggestion that growth hormone's effects were mediated by a 'sulphation factor', rather than by growth hormone directly. Subsequent purification of this activity led to the characterisation of two polypeptides named somatomedin C and somatomedin A (Daughaday et al., 1972). These findings formed the basis for the 'somatomedin hypothesis' of GH action. Although findings that GH was able to directly stimulate cartilage growth by Isaksson et al. (1985) cast doubt over the validity of this hypothesis, it has now been shown that these direct effects of GH may be due to local production of IGF-I in the cartilage (Schlechter et al., 1986). Numerous other studies have supported and strengthened this hypothesis including the observation of increased growth in hypophysectomised rats following administration of recombinant IGF-I (Schoenle et al., 1982).

In 1972, Pierson and Temin isolated the factors in calf serum responsible for its mitogenic actions on cultured cells. These low molecular weight factors were named 'multiplication stimulating activity' (MSA), since when added to culture medium, MSA stimulated cells to replicate. It was later demonstrated that MSA was produced and secreted by rat liver cells in culture (Dulak et al., 1973). This activity was subsequently purified to homogeneity and it is now recognised that MSA is the rat equivalent of IGF-II (Marquardt et al., 1981)

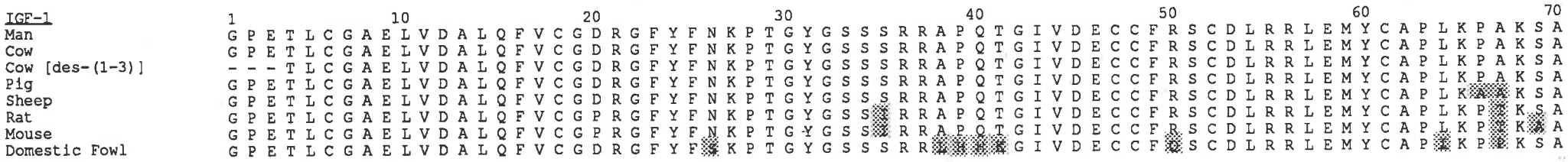
The third line of investigation that led to the discovery of the IGFs was made by researchers examining the insulin-like metabolic effects of serum on insulin target tissues such as muscle and adipose tissue. The insulin content of serum alone was unable to account for the magnitude of the observed effects suggesting the presence of other factors. When it was found that these insulin-like effects were not able to be suppressed by anti-insulin antibodies, it indicated that there were indeed immunologically distinct insulin-like substances in serum (Froesch et al., 1963). In 1976, Rinderknecht and Humbel purified this 'non-suppressible insulin-like activity' (NSILA) from tonne quantities of human serum and discovered that the activity resided in two distinct peptides. Finally purification and sequence analysis of these peptides was performed and the peptides named insulin-like growth factor-I and insulin-like growth factor-II (IGF-I and IGF-II) (Rinderknecht and Humbel, 1978). It was subsequently shown that Somatomedin C was identical to IGF-I (Svoboda et al., 1980).

1.5.2 Structure of the IGFs

Both IGF-I and IGF-II peptides exhibit 45-50 % similarity to insulin and thus belong to a peptide family which includes insulin, relaxin and nerve growth factor. Also both are single chain polypeptides, consisting of 70 and 67 amino acids repectively cross-linked by three intrachain disulphide bridges. Like pro-insulin both peptides contain an N-terminal B domain and an A domain that are linked by a connecting peptide or C domain. However, both peptides unlike pro-insulin contain an additional carboxy-terminal extension region (D domain). A further E extension peptide is also coded for by IGF-I genes (Jansen et al., 1983; Rotwein et al., 1986) but is not present on circulating forms of the peptide, due to proteolytic modification (Daughaday and Rotwein, 1989). The three disulphide bonds within IGF-I, IGF-II and insulin are in identical positions in all three molecules suggesting evolutionary relatedness (Rinderknecht and Humbel, 1978a). Furthermore, most of the

Figure 1

Comparative sequence data of insulin-like growth factor-I peptides across species



Comparison of the amino acid sequences of IGF-I from several species. Residues which differ between species are indicated with a darkened box. The data is from Jansen et al., (1983) human, Bell et al., (1986) mouse, Shimatsu and Rotwein (1987) rat, Kajimoto and Rotwein (1989) chicken Wong et al., (1989) sheep, Brem et al., (1990) pig and Francis et al., (1986) cow.

hydrophobic residues of the A and B domains are also identical suggesting a similar tertiary structure for this family. Thus a model for the tertiary structure of the IGF-I was proposed based on that of porcine insulin (Blundell et al., 1983) This observation has been essentially confirmed with the determination of the solution structure of IGF-I using nuclear magnetic resonance (Cooke et al., 1991)

The primary structure of the IGFs from numerous mammalian (human, rat, mouse, bovine, porcine and ovine) and several non-mammalian species (chicken, Xenopus, hagfish and salmon) have been characterised from either the protein or cDNA sequence (Rinderknecht et al., 1978; Bell et al., 1986; Honegger et al., 1986; Murphy et al., 1987; Tavakkol et al., 1988; Francis et al., 1989 ; Shimatsu et al., 1987; Stempian et al., 1986; 1992; Kajimoto et al.,1989, 1990; Nagamatsu et al., 1991; Duguay et al., 1992). Within the mature IGF-I peptide, 76 % of the amino acids are conserved amongst all vertebrate species examined thus far (Ward et al., 1993). Figure 1 shows the high degree of conservation throughout species. The importance of the B domain in binding to serum binding proteins (DeVerode et al., 1985; Joshi et al., 1985; Szabo et al., 1988) is reflected by its highly invariant nature across species.

In addition to the small species differences observed, variant forms of IGF-I and IGF-II have also been identified. An IGF-I molecule lacking the first three amino-terminal amino acids has been purified from bovine colostrum (Francis et al., 1986) and human brain (Sara et al., 1986; Carlsson-Skwirut et al., 1986). Large forms of IGF-2 have been isolated from human serum (Zumstein et al., 1985).

1.5.3 IGF-I gene structure, expression and regulation

The genes for IGF-I which have been studied most extensively in both the human and rat, contain at least 5 exons and generally encode two precursor forms of IGF-I. The human and rat genes extend over 80 kb of DNA . The human IGF-I locus has been mapped to the long arm of chromosome 12 (Brisenden et al., 1984). Five of the six exons found in the rat gene are analogous to the human genes, in both organisation and sequence homology (Shimatsu et al., 1987a). From these genes two types of IGF-I precursorsof 153 amino acids (type a)) and 195 amino acids (type b)) arise via the combined use of multiple initiation sites, alternative splicing events and variable polyadenylation leading to the generation of multiple mRNA species in both species (Rotwein et al., 1991). The same situation occurs in the mouse (Bell et al., 1986).

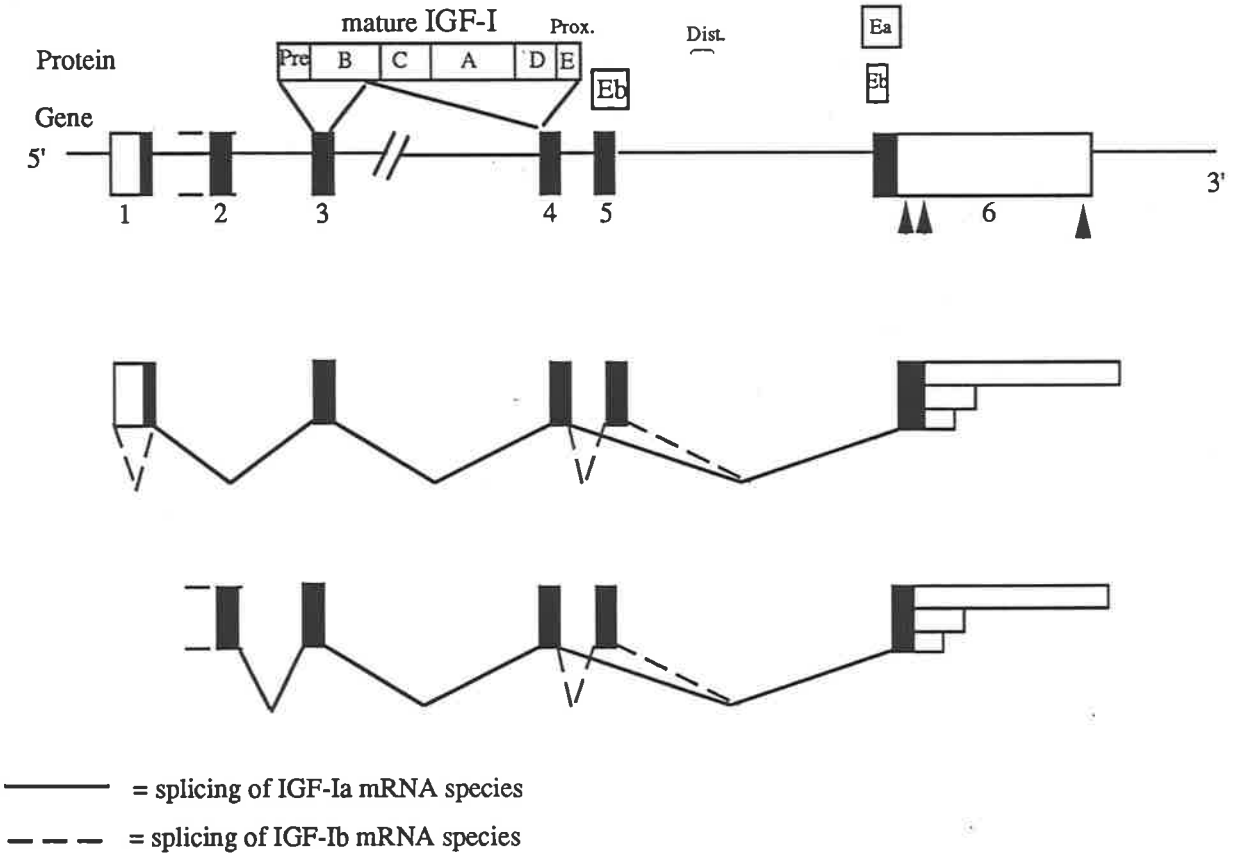
The two precursor molecules while containing the same B, C, A and D domains, differ in the distal end of the carboxy terminal E domain (the proximal end of the E peptide is also identical). The IGF-Ia) molecule possesses a 19 amino acid distal E peptide resulting in an E peptide of 35 amino acids, while in IGF-I b) there are an additional 61 amino acids giving rise to a 77 amino acid extension. Athough there is a high degree of nucleotide conservation between the rat and human IGF-I a) precursor, a significant divergence exists with IGF-Ib) (Sara et al., 1990). Also, the splicing mechanism by which the IGF-I b) species is generated differs between the human and rat.

In the case of the human gene, the domains conserved between IGF-I a) and IGF-I b) (B, C, A and D) are encoded by exons 2, 3 and 4 while the divergent E regions are composed of either exon 6 (for IGF-I a)) or exon 5 (for IGF-I b)). In rodents the IGF-I a) precursor is composed of exons 1 (or 2), 3, 4 and 6 in a similar fashion to the human case (Exon 2 is likely to encode a variant amino terminus for the signal peptide of IGF-I in the rat). However, the IGF-I b) precursor is composed of exons 1, 3, 4 and sequences from both exon 5 and 6 (See figure 2). This difference is due to a splice donor sequence which is present in exon 5 of the rat gene but not in its human counterpart (Lowe et al., 1988).

In addition to the alternative splicing that generates the pre-pro-IGF-I molecules, there are also multiple polyadenylation sites 3' of the IGF-I genes which result in further complexity of the IGF-I mRNA population. This phenomenon has been best studied in rat , where the heterogeneity of IGF-I mRNA species was found to be largely due to varying lengths of 3' untranslated region (Lund et al., 1989) Interestingly, evidence for multiple polyadenylation recognition sequences has been found for rat, mouse, and human genes (Shimatsu et al., 1987; Bell et al., 1986; LeBouc et al., 1986).

Additionally, in generating the IGF-I protein there are three potential initiation methionine (AUG) codons encoded by the gene. It is currently not known which of these initiation codons in used *in vivo* although initiation from the met at -48 has been observed for an *in vitro* translation system (Rotwein et al., 1987). Also mRNA species with different 5' untranslated regions can arise due to initiation of transcription at multiple sites around exon 1 and 2, as is the case for the rat (Roberts et al., 1987; Shimatsu et al., 1987a; Adamo et al., 1989). Of relevance to this finding is the study of the tandem promoter regions in the rat IGF-I gene showing that there are no TATA or CAAT motifs and that initiation occurs at multiple sites within each promoter region (Adamo et al ., 1991; Hall et al., 1992). In its

Figure 2 Structure and splicing of the rat IGF-I gene



Schematic diagram of the rat IGF-I gene and its mRNAs. This diagram is not drawn to scale and has been adapted from Hall et al., (1992). LeRoith et al., (1991) and Ward et al., (1993). Exons 1-6 are indicated by boxes with coding regions shown in black and non-coding regions indicated in white. The dashes 5' of exon 2 indicate that the full extent of untranslated region for this exon is still unknown. Three polyadenylation sites in exon 6 have beed indicated with arrow heads. The protein coding capacity of the exons is indicated above the gene .

human counterpart, a single promoter region also lacking both of these elements has been reported (Woon-Kim et al., 1991).

IGF-I mRNA is expressed at low levels in most organs and tissues (liver, kidney, skeletal muscle, intestine , ovary, testes, brain, adipose) during mammalian growth and development (Lund et al., 1986; Han et al., 1987; Beck et al., 1987; Roberts et al., 1987; Bondy et al., 1990). Lower levels of IGF-I are generally present in fetal tissues (here IGF-II expression predominates). However, the liver is the major site of expression and synthesis during postnatal growth (Steele et al., 1989). Here, as in other tissues IGF-I expression is increased by GH (Roberts et al., 1986; Mathews et al., 1986; Norstedt et al., 1987). The mechanisms by which GH is able to induce transcription and alter splicing of the IGF-I mRNAs are unknown.

While endocrine production of IGF-I from the liver is GH-dependent, in many extrahepatic tissues autocrine/ paracrine production of IGF-I may be regulated by local factors (among them hormones such as glucocorticoids, thyroid hormone and estrogen).

Nutritional status is also a very important regulator of IGF-I expression. In rats fasting has been shown to reduce hepatic IGF-I mRNA levels and subsequently IGF-I serum levels (Emler et al., 1987; Straus et al., 1990). Similar results have been observed in human malnutrition and fasting (Phillips et al., 1984; Ho et al., 1988).

When total RNA is subject to Northern analysis for IGF-I, multiple mRNA species are detected (as mentioned above) which range between 0.8 kb-7.5 kb (Rotwein et al., 1986; Casella et al., 1987). In many cases specific rat IGF-I transcripts have been shown to be differentially regulated by GH (Lowe et al., 1987; Hernandez et al., 1989), nutritional status (Adamo et al. ,1991), and to vary in abundance between different tissues (Hoyt et al., 1988; Adamo et al., 1989). Thus the complexity of IGF-I production provides ample room for regulation at many levels, e.g. choice of promoter, splicing, polyadenylation, translation , proteolytic modification and even post translational modifications such as glycosylation (Bach et al., 1990)

1.5.4 Actions of IGF-I (*in vitro and in vivo*)

By virtue of its structural similarities to insulin it is not suprising that IGF-I (and IGF-II) is able to elicit qualitatively similar biological responses. These acute, metabolic actions of IGF-I are mediated via the insulin receptor on target tissues such as adipose and via the IGF-I receptor in muscle (Zapf et al., 1978). In adipose tissue these actions include

stimulation of glucose transport as well as increased lipid, glycogen and protein synthesis. Furthermore, lipid, glycogen and protein breakdown are concurrently inhibited with 50-100 fold lower potency than insulin (Zapf et al., 1978).

The longer term or growth promoting effects of IGF-I have been observed extensively using cultured cells or organ explants. In these studies IGF-I has been repeatedly shown to stimulate RNA, DNA synthesis, protein synthesis and cell proliferation while inhibiting protein degradation (for review see Froesch et al., 1985). IGF-I has been postulated as a 'progression factor' which stimulates cells through the DNA synthesis phase of the cell cycle and acts in concert with competence factors such as PDGF and FGF (Stiles et al ., 1979)

In addition, IGF-I has been shown to play a role in cell differentiation. Schmid et al .(1983) showed that IGF-I enhanced the differentiation of chicken embryo fibroblasts into muscle cells. Other studies have since shown stimulation of differentiation in osteoblasts, myoblasts, chondrocytes, adipocytes and oligodendrocytes (Schmid et al., 1984; Bhaumick et al., 1991; Florini et al., 1986; Kato et al., 1980; Smith et al., 1988; McMorris et al., 1986; Mozell et al., 1991) confirming this role. Thus in addition to its metabolic role, IGF-I is an important stimulator of cellular replication and differentiation. It should be noted that these growth promoting actions *in vitro* depend upon the state of the target cell , the presence of appropriate cellular receptors for IGF-I/ insulin and the regulatory actions of IGF binding proteins (see later section).

Due to accumulating data on the production (D'Ercole et al., 1984) and actions of IGF-I in non-hepatic tissues (Schlechter et al., 1986), the original somatomedin hypothesis has now been modified to incorporate the paracrine and autocrine actions of this peptide as well as those which occur due to endocrine release in response to GH (Holly et al., 1989).

Evaluation of IGF-I's growth promoting actions has also been made *in vivo*. As with the *in vitro* studies both short term 'metabolic' and longer term 'growth promoting' effects of IGF-I administration have been seen. The short term insulin-like metabolic effects have been observed in the form of hypoglycaemia when IGF-I is administered as an intravenous bolus to rats (Zapf et al., 1986) , mini-pigs and healthy humans (Guler et al., 1986). This effect is due to the presence of 'free' IGF-I which is not associated with binding proteins. The hypoglycaemic effect may be overcome by chronic subcutaneous infusion of the peptide which allows co-ordinate induction of IGF carrier proteins. Such infusions stimulated body weight gain , longitudinal bone growth and accretion of lean body

mass in both normal and growth retarded rodents (Schweiller et al., 1986; Hizuka et al., 1986; Van Buul-Offers et al., 1988; Tomas et al., 1990, 91, 91a, 92, 93). Furthermore, biosynthetic IGF-I has been shown to increase the body weight and tail length of Snell Dwarf mice, deficient in the production of growth hormone and thyroid-stimulating hormone (Van Buul-Offers et al., 1986). Similarly long-term subcutaneous infusion of recombinant IGF-I also stimulated the growth of hypophysectomised rats (Skottner et al., 1987; Guler et al., 1988), neonatal rats (Phillips et al., 1988) and a GH-deficient strain of dwarf rat (Skottner et al., 1989).

It should be noted that although IGF-I infusion stimulates growth, in most studies the response is quantitatively smaller than that with GH. Also IGF-I treatment may exert its effects on different tissue targets than GH. In hypophysectomised rats IGF-I selectively stimulated the growth of kidney, spleen and thymus while GH had more pronounced effects on skeletal muscle (Guler et al., 1988). Furthermore, in transgenic mice expressing IGF-I in the absence of GH, selective growth was seen in the brain compared with liver enlargement in GH transgenics (Mathews et al., 1988; Behringer et al., 1990). These differences in response to IGF-I and GH may be partially due to the changes these respective treatments produce on the IGFBPs.

Apart from stimulating growth in normal and hormone deficient rodents, exogenous IGF-I has been shown to stimulate erythropoiesis in normal or neonatal rats (Kurtz et al., 1988; Phillips et al., 1988) and improve renal function in rats and man (Martin et al., 1991; Guler et al., 1989). Additionally, IGF-I has been found to reduce weight loss associated with starvation, diabetes or glucocorticoid treatment of rodents (Skottner et al., 1987; Tomas et al., 1992, 1993). Finally, IGF-I has been shown to act in concert with other growth factors to induce wound healing in pigs (Lynch et al. ,1989) and IGF-I expression has been strongly associated with wound chambers in rats and rabbits during healing (Spencer et al., 1988a; Mueller et al., 1991; Suh et al., 1992).

1.5.5 IGF Receptors

IGF-I and IGF-II act through cellular receptors as is the case with most polypeptide hormones. On the basis of physical studies, (indicating structural features), and on cross-reactivity studies there have been two receptors recognised for binding of IGF-I and IGF-II respectively, in addition to the insulin receptor through which some IGF-I and IGF-II

actions may be mediated (see figure 3). However, this classification may be an oversimplification of the *in vivo* situation.

The type 1 receptor binds IGF-I with higher affinity than IGF-II and migrates with a molecular weight of 300 kDa under non-reducing conditions, although due to glycosylation its mature size is over 350 kDa (Rechler and Nissley, 1985). The primary structure of this protein has been deduced from its cDNA sequence and has been shown to be highly homologous to the insulin receptor (Ulrich et al., 1986).Thus the cDNA encodes a single transmembrane spanning polypeptide composed of two subunits (α and ß)

As with the insulin receptor the mature type 1 receptor is heterotetrameric and composed of two extracellular α (ligand binding) subunits and two transmembrane-spanning ß (signal-transducing) subunits in an $\alpha_2ß_2$ configuration. While the approximately 130 kDa α subunits (80 kDa from the cDNA clone) bind IGF-I, they also bind IGF-II to a lesser extent and bind insulin weakly. The intracellular regions of the approximately 95 kDa, the ß subunits (71 kDa from cDNA), have no binding capacity but have intrinsic tyrosine kinase activity, responsible for signal transduction (Morgan et al., 1986). Despite their similar structures it is likely that the type 1 receptor and the insulin receptor have distinct signalling capabilities (Lammers et al., 1989)

Other forms of the type 1 receptor have been identified that differ in their affinity for the IGFs. In human placenta evidence was found for the presence of binding sites on type 1 receptors which preferred IGF-II over IGF-I (Casella et al., 1986). Furthermore, on IM9 lymphocytes a 130 kDa α subunit has been identified which also prefers IGF-2 and insulin equally over IGF-I (Jonas et al., 1990). To add confusion, it has also been demonstrated that insulin α-ß chains and/type 1 α-ß chains form receptor hybrids *in vitro* and *in vivo* (Treadway et al., 1989; Moxham et al., 1989; Soos et al., 1989). The physiological role of these receptors is presently unknown as is their contribution to the total receptor pool . It is likely they provide a further level of diversity for IGF-I and insulin action by virtue of different signalling capabilities in reponse to ligand binding.

The mechanism of signal transduction from the type 1 receptor is yet to be fully characterised. One likely pathway for this transduction is via phosphorylation of intracellular proteins in response to ligand binding. A common phosphorylated substrate (IRS-1) has been identified following both insulin receptor and type 1 receptor binding, suggesting both receptors may have similar or common signal transduction pathways (Myers et al., 1993). However the subsequent role of phosphorylated IRS-1 has yet to be shown.

In contrast to the type 1 receptor, the type 2 receptor does not bind IGF-I or insulin (Kornfeld, 1992). Furthermore, it is a monomeric transmembrane spanning protein of approximately 260 kDa which unlike the type 1 receptor lacks intrinsic kinase activity. Also, there is little evidence to conclusively show that binding of IGF-II to this receptor consistently propagates an intracellular signal.

Based on molecular cloning and subsequent physical studies it was shown that the human form of the type 2 receptor was identical to the cation-independent mannose 6-phosphate receptor (Morgan et al., 1987; Roth et al., 1987; Tong et al., 1988; Kiess et al., 1988). This observation can be extended to all mammals thus far studied but has not been observed in non-mammalian species (Oshima et al., 1988; Canfield et al., 1989) (A receptor identified in chicken liver possesses a binding site for Mannose-6-phosphate but lacks an IGF-II binding). Hence, the type 2 receptor contains a large extracellular domain with distinct binding sites for both mannose 6-phosphate (M6P) moieties and IGF-II (Braulke et al., 1988).

The large extracellular domain of the type 2 receptor is composed of 15 contiguous repeats of approximately 150 amino acids each (Lobel et al., 1988). Despite the presence of spatially distinct binding sites for both IGF-II and M6P, it has been shown that binding of either ligand may inhibit binding of the other, by some as yet undetermined means (Kiess et al., 1988,1990; Nolan et al., 1990). Intracellularly the type 2 receptor exists on the Golgi membranes where it binds to the M6P moieties (at neutral pH) of newly synthesised lysosomal enzymes. These enzymes are then released under acidic conditions in the late endosome compartment of the Golgi. The type 2 receptors on the cell surface are in equilibrium with the cellular pool and experimental evidence suggests a constant cycling of these receptors independently of receptor occupancy (Braulke et al., 1987). Besides effects on cellular enzymes, the type 2 receptor has been shown to mediate the degradation of extracellular IGF-II by receptor internalisation (Kiess et al., 1987). Thus, the function of such a protein in relation to IGF-II action is still incomplete.

Few reports have conclusively shown signalling by this receptor in response to IGF-II binding using receptor antibodies. Most reports have relied upon the fact that IGF-II was more efficient in stimulating a particular response than IGF-I. However, antibodies to the type 2 receptors of Hep-G2 human hepatoma cells and Balb/c 3T3 cells have been shown to mimick the stimulation of glycogen synthesis and DNA synthesis respectively observed with IGF-II in these cells (Hari et al., 1987; Kojima et al., 1988). Moreover, by examining the

Ca^{++} influx which occurs in Balb/c 3T3 cells primed with PDGF and EGF prior to IGF-II stimulation , it has been possible to show a coupling of the type 2 receptor with a GTP binding protein in generating this response (Nishimoto et al., 1989).

Interestingly, naturally occurring mouse mutations (deletions) of chromosome 17 lead to a lethal phenotype when inherited from the mother. The absence of a gene called *Tme* (T-associated maternal effect) in these embryos has been suggested to cause this lethality. Upon mapping of candidate genes falling within these deleted regions and expression studies of these genes, it has been deduced that the type 2 receptor is the cause of this mutation (Barlow et al., 1991). Thus, the type 2 receptor is paternally imprinted in mice with expression only occurring if a functional allele is inherited from the mother. Conversely, the IGF-II ligand itself is imprinted (see section 1.6.2) such that expression occurs only if the IGF-II gene is inherited from the father (DeChiaria et al., 1991). The significance of these findings is as yet unknown but it has been speculated that the function of the type II receptor is to limit embryonic growth by acting as a 'sink' for IGF-II. Thus while it is in the paternal interest to maximise the growth of offspring it is in the maternal interest to limit the size of her offspring such that her welfare is not compromised by the growth of the litter, through competition for scarce maternal resources (Haig and Graham, 1991; Willison, 1991).

1.5.6 The Insulin-like growth factor Binding Proteins (IGFBPs)

The IGFBPs represent a family of proteins which specifically bind the IGFs but are structurally unrelated to either of the high affinity IGF receptors. The IGFBPs , determine their availability of the IGFs and are capable of regulating their biological actions. Thus far six classes of IGFBP have been identified (called IGFBP-1 to IGFBP-6 according to their order of discovery) which vary in their size, proteolytic modification and preference for binding IGF-I and IGF-II. Despite differences between each class of IGFBP, all are found to have a high degree of primary amino acid sequence homology. The highly invariant nature of cysteine residues present in two clusters at both the N-terminus and C-terminus of all IGFBPs so far elucidated suggests that all are likely to have extensive secondary structure homology as well. Furthermore, these structural similarities are observed between species implying a strong conservation of function exists in this family of proteins. As extensive reviews of this field have been published within recent years (Baxter and Martin, 1989;

Rechler and Nissley, 1990; Lamson et al., 1991; Rechler and Brown, 1992; Baxter, 1993 and Rechler, 1993) I shall only cover each class of binding protein briefly.

1.5.6.1 IGFBP-1

The isolation and characterisation of IGFBP-1 occurred as the result of work on proteins purified from human amniotic fluid (Drop et al., 1979), conditioned media from human (Hep G2) hepatoma cells (Moses et al., 1983; Povoa et al., 1985) and from human placental tissue (Koistinen et al., 1986). It has also been isolated from the conditioned medium of rat H4IIE hepatoma cells, where it is the major IGFBP (Yang et al., 1990). In all cases a protein of between 28-35 kDa was identified. The size of this BP has been deduced as 25 kDa from its cDNA sequence (Brewer et al., 1988). Analysis of its sequence showed IGFBP-1 has 5 potential O-linked glycosylation sites suggesting that glycosylation differences may account for the initial size heterogeneity observed among separately purified species. IGFBP-1 also contains an Arg-Gly-Asp (RGD) motif near its carboxyl terminus. Such motifs are thought to enable adhesion of proteins to the cell surface via integrins and other cell surface proteins.

The cDNA s of both rat and human species have now been characterised and show extensive homology (Brewer et al., 1988; Brinkman et al., 1988; Lee et al., 1988; Murphy et al., 1990). In addition, the rat gene has been isolated and shown to contain four exons spanning approximately 5 kb of DNA. Analysis of the promoter region of the rat IGFBP-1 gene has identified homology with the insulin-response element of the rat phosphoenolpyruvate (PEPCK) gene (Unterman et al., 1992). Hence it is not surprising that IGFBP-1 is negatively regulated by insulin (Ooi et al., 1990). IGFBP-1 gives rise to a 1.4-1.5 kb species upon Northern blotting. An AUUUA (mRNA instability) sequence has been identified in the 3' untranslated region of this mRNA, consistent with the short half-life of this species (Luthman et al., 1989; Orlowski et al., 1990).

While IGFBP-1 is found in body fluids such as lymph and milk its major source is believed to be the liver. Serum levels of IGFBP-1 exhibit a diurnal rhythm which is independent of GH secretion (Baxter and Cowell, 1987). However, serum levels of IGFBP-1 fluctuate in response to dietary intake suggesting some hormonal control. Glucagon or glucocorticoids increase, while insulin decreases serum IGFBP-1 levels (Hilding et al., 1992; Lee et al., 1992). Furthermore, in rats, hepatic IGFBP-1 transcription is inhibited by insulin and stimulated by dexamethasone (Luo et al. ,1990; Ooi et al., 1990;

Orlowski et al., 1990). These expression changes are also observed in cultured hepatocytes, along with increased transcription following administration of agents which increase intracellular cAMP (eg glucagon) (Unterman et al., 1991; Lewitt et al., 1989). In these respects control of IGFBP-1 expression mimicks control of the gluconeogenic enzyme PEPCK (Magnuson et al., 1987), leading to the suggestion that IGFBP-1 plays a major role in maintenance of glucose homeostasis (Lewitt and Baxter, 1991). Thus, it is thought that metabolic fluctuations in IGFBP-1 could parallel increases in IGF-I and inhibit the insulin-like effects of free IGF-I. Support for this suggestion comes from the ability of exogenously administered hIGFBP-1 to block the hypoglycaemia observed in rats given exogenous IGF-I (Lewitt et al., 1991).

1.5.6.2 IGFBP-2

IGFBP-2 was originally purified from serum-free medium conditioned by rat liver BRL 3A cells (Mottola et al., 1986) and MDBK bovine kidney cells (Szabo et al., 1988). The protein sequence of rat IGFBP-2 has been determined, as has the N-terminal sequence of IGFBP-2 purified from human serum (Brown et al., 1989; Zapf et al., 1990).

Complementary DNA clones for rat, human, porcine and bovine IGFBP-2 have been isolated (Brown et al., 1989; Binkert et al., 1989; Delhanty et al., 1992; Upton et al., 1990). From these sequences it has been deduced that IGFBP-2 has a molecular weight of approximately 30 kDa. This size is inconsistent with IGFBP-2's estimated molecular weight from SDS-PAGE, of approximately 40 kDa. under reducing conditions and 34 kDa. under non-reducing conditions. Sequence analysis also showed the absence of glycosylation signals for IGFBP-2. Hence this size disparity is likely to be due to some other post translational modification or structural feature of the mature protein. All 18 conserved cysteines are present in mature BP-2 (Rechler et al., 1993). Also IGFBP-2, like IGFBP-1 contains an RGD motif near its carboxy-terminus. IGFBP-2 has been shown to have a higher affinity for IGF-II than IGF-I (Roghani et al., 1991; Forbes et al., 1988; Rechler et al., 1993)

A single mRNA species of 1.5-1.6 kb encodes IGFBP-2 and is expressed abundantly in many tissues of the fetal rat (eg. liver, stomach, brain, kidney and lung) as well as the adult with a different distribution (mainly brain, testes, ovary, kidney and less abundant but present in liver) (Margot et al., 1989; Ooi et al., 1990). Also IGFBP-2 has

been found to expressed in early rat embryonic tissue at sites distinct from IGF-II expression suggesting a possible role in development independent of IGF-II (Wood et al., 1990).

IGFBP-2 appears to increase in states where there is insufficient IGFBP-3 to bind available IGF pools, suggesting that IGFBP-2 acts largely as an alternative carrier for the IGFs (Baxter et al., 1993). Furthermore, IGFBP-2 levels are influenced by GH status. Thus BP-2 levels are increased at both the mRNA and protein levels in hypophysectomised rats (Orlowski et al ., 1990). However, GH alone fails to decrease IGFBP-2 levels to normal suggesting that the regulation of this protein is also dependent on other factors. One such factor is likely to be nutrition. Fasting leads to an elevation of IGFBP-2 protein and hepatic mRNA in rats (Ooi et al., 1990; Orlowski et al., 1990). Interestingly, the brain and kidney levels of IGFBP-2 are not altered by fasting indicating a possible mechanism for retaining IGF in these organs (Straus and Takemoto, 1990; Tseng et al., 1992). An additional, prominent regulator of IGFBP-2 is insulin. Hepatic IGFBP-2 has been shown to be increased 10-20 fold in streptozotocin-treated rats (Ooi et al., 1990). This increase could be reduced by administration of insulin (Boni-Schnetzler et al., 1989). Also serum IGFBP-2 was found to be elevated in transgenic mice which overexpressed IGF-I in the presence or absence of normal GH levels suggesting that IGF-I alone may be a regulator of IGFBP-2 (Camacho-Hubner et al., 1991). However it is possible that in these animals IGFBP-2 is elevated as a consequence of a decreased circulating level of insulin.

1.5.6.3 IGFBP-3 and the ternary complex

Approximately 75 % of the IGF pool (IGF-I + IGF-II) in the adult rat circulation exists as a 150 kDa , ternary complex readily identified upon neutral gel filtration chromatography of plasma (Baxter et al., 1986). A similar situation has been observed in humans (Gargosky et al., 1991). This complex is composed of IGFBP-3, a 40-45 kDa glycoprotein (ß- subunit), one molecule of either IGF-I or IGF-II (γ-subunit) and an 84-86 kDa. glycoprotein termed the acid-labile subunit (ALS, α-subunit) (Baxter et al., 1989). Sugar residues on both BP-3 and ALS contribute significantly to the size of the overall complex. This complex prolongs the half-life of IGF-I and IGF-II in the circulation (Lewitt et al., 1993) and may be too large to pass from the blood into the surrounding tissues (Martin and Baxter, 1992). The binding of IGF-I or -II to IGFBP-3 is independent of ALS binding but binding of ALS to the complex is absolutely reliant upon prior binding of IGF (Baxter et al., 1989). The IGFBP-3 portion of the ternary complex has been shown to be

susceptible to proteolytic cleavage which can release already bound IGFs or alter affinity of the complex for re-uptake of IGFs (Clemmons et al., 1983; Guidice et al., 1990; Hossenlopp et al., 1990; Davenport et al., 1992). Thus proteolysis may provide a mechanism for IGF bioavailability in certain states.

IGFBP-3 has been purified from human, rat, porcine and bovine serum and its amino acid sequence determined (Rechler et al., 1993). The mature protein has an apparent molecular weight between 34-45 kDa and has several sites for N-linked glycosylation (Zapf et al., 1988). Rat IGFBP-3 often appears as a triplet of 43, 41 and 39 kDa on ligand blots, probably due to glycosylation differences between these (iso)forms (Hossenlop et al., 1987).

Complementary DNA sequences have been attained for the human (Wood et al., 1988), rat (Albisten and Herington, 1990), bovine (Spratt et al., 1991) and porcine (Shimasaki et al., 1990) proteins. These sequences showed that all 18 conserved cysteines were present and unlike the smaller binding proteins no RGD motifs were present. The human gene for IGFBP-3 contains 4 protein coding exons and a fifth exon encoding a long 3' untranslated region (Cubbage et al., 1990). The gene spans 8.9 kb of DNA and in both human and rat a single mRNA of approximately 2.5 kb is produced (Wood et al., 1988; Shimasaki et al., 1989). In the rat expression is observed in a variety of tissues with the kidney and liver being the most prominent (Takenaka et al., 1991). While IGFBP-1 and -2 are expressed in numerous tissues, the relatively small range of IGFBP-3 expressing tissues is consistent with the role of this protein as the major carrier of endocrine IGF-I.

The acid labile subunit (ALS) has been purified and its cDNA isolated from a human liver library (Leong et al., 1992). A glycosylated protein of approximately 64 kDa was deduced from DNA sequence data, somewhat smaller than its 84 kDa size by SDS PAGE. The protein contains a series of highly leucine rich repeats, thought to be involved in protein-protein interactions (Rechler et al., 1993).

Serum IGFBP-3 and ALS levels follow the same ontogeny as IGF-I, being lowest in the neonate and steadily increasing to maximum levels at puberty before declining from puberty onwards (Baxter , 1990). A major regulator of both IGFBP-3, and ALS, and hence ternary complex formation is GH. Levels of IGFBP-3 and ALS are increased under conditions of GH excess and decreased under GH-deficient conditions. In hypophysectomised rats and GH-deficient transgenic mice, serum IGFBP-3 as shown by ligand blotting was reduced (Zapf et al., 1989; Clemmons et al., 1989; Camacho-Hubner et

al., 1991). The reduced levels could be increased by infusions of GH, IGF-I or overexpression of IGF-I in the GH-deficient transgenic mice , suggesting that IGF-I rather than GH is the primary regulator of IGFBP-3 in rodents (Interestingly, changes in plasma IGFBP-3 in response to hypophysectomy were not reflected by changes in BP-3 hepatic mRNA (Albiston and Herington, 1992), suggesting that regulation of BP-3 expression may be post-transcriptional). In contrast to these findings it has been shown that GH directly regulates ALS expression (Zapf et al., 1989).

IGFBP-3 levels are also nutritionally regulated, but to a lesser extent than IGFBP-1 or IGFBP-2 levels. There does not seem to be any diurnal rhythm associated with IGFBP-3 levels (Baxter and Martin, 1989). Although fasting of rats for 2 days did not significantly lower BP-3 levels, protein deprivation resulted in significant decreases of IGFBP-3 levels and reversal of this trend was observed following either GH or IGF-I infusion (Orlowski et al., 1990; Clemmons et al., 1989).

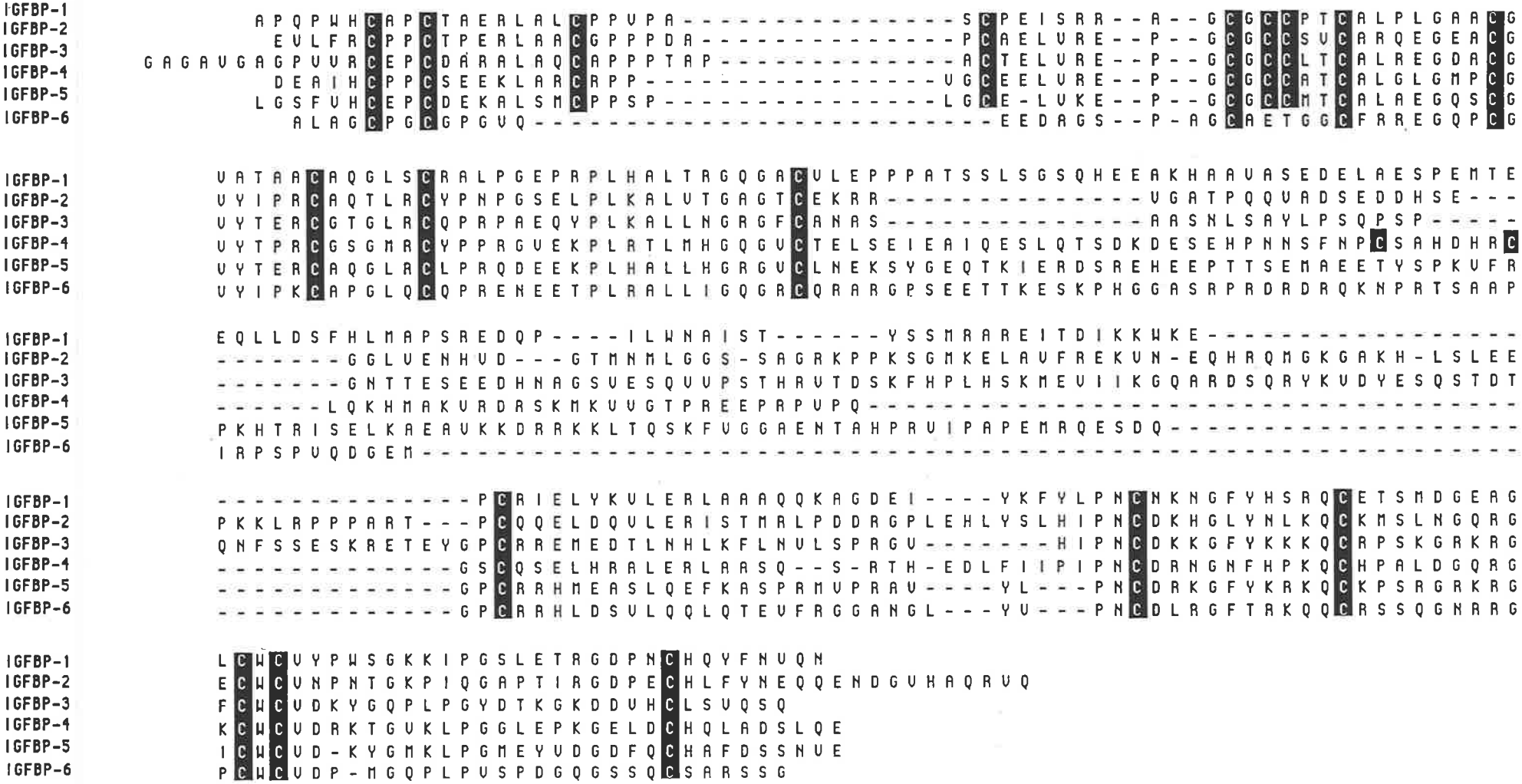
Other factors affecting IGFBP-3 levels are disease states such as chronic renal failure and diabetes mellitus which lead to increased and reduced levels respectively (Baxter and Martin , 1986; Clemmons et al., 1989). During pregnancy in the rat the operation of a specific IGFBP-3 protease is responsible for a pronounced loss of the 150 kDa complex despite the normal synthesis of BP-3 at this time (Gargosky et al., 1990; Donovan et al., 1991).

1.5.6.4 IGFBP-4, -5 and -6

In contrast to the previously discussed binding proteins (IGFBP-1,-2 and -3) relatively little is known about the regulation and roles played by these distinct classes of binding proteins.

A low molecular weight binding protein of approximately 24 kDa., IGFBP-4 has been purified from rat serum (Shimonaka et al., 1989), porcine serum (Walton et al., 1990) and the conditioned media of a human osteoblast cell line (Mohan et al., 1989). Furthermore, cDNAs of this protein has been isolated from rat liver, human placenta and TE89 osteosarcoma cell lines (Shimasaki et al., 1990a; La Tour et al., 1990). While IGFBP-4 contains the 18 cysteines conserved in most other binding proteins it is unique by virtue of two extra cysteines. In addition this binding protein has sites for N-linked glycosylation but no RGD sequence is present. The 2.6 kb mRNA species which encodes BP-4 is expressed in many tissue of the rat including liver where there are the highest levels,

Figure 3 Sequence Alignment of Cloned Rat IGF-Binding Proteins



adrenals, testis, spleen, heart, lung, kidney, stomach and brain (Rechler et al., 1993). In osteoblast cells the production of this binding protein has been shown to be stimulated by parathyroid hormone suggesting that *in vivo* it may be specifically involved in bone development or remodelling (La Tour et al., 1990; Torring et al., 1991).

IGFBP-5 has been purified from adult rat serum and human cerebral spinal fluid (CSF) respectively (Shimasaki et al., 1991a; Binoux et al., 1991) With the cDNA cloning of the rat and human genes IGFBP-5 was deduced to be approximately 28.5 kDa (Shimasaki et al., 1991). It contains all 18 conserved cysteines and lacks both glycosylation and an RGD motif. Unlike the other IGFBPs,IGFBP-5 produces a large 6 kb transcript which predominates in rat kidney but is also present at high levels in lung, heart, stomach, adrenal and intestine (Shimasaki et al., 1991). The protein has similar affinities for both IGF-I and -II.

More recently a sixth class of IGFBP has been identified and purified from human lung fibroblasts (Forbes et al., 1990; Martin et al., 1990) with molecular weight between 30-34 kDa.. This protein has also been identified in human and rat serum (Zapf et al., 1990; Shimasaki et al., 1991). From sequence analysis and deductions from the cDNA , IGFBP-6 contains an N-terminus which is quite distinct from that conserved in IGFBP1-5. Also IGFBP-6 lacks 2 and 4 of the 18 conserved cysteines in the rat and human proteins respectively (Rechler et al. ,1993). As with IGFBP-5, IGFBP-6 lacks the RGD motif. IGFBP-6 is also glycosylated (Forbes et al., 1990). Interestingly, this IGFBP shows a marked preferntial affinity (60-70 fold) for IGF-II over IGF-I (Rechler et al., 1993). A mRNA of approximately 1.3 kb is produced for this protein and expression is widely distributed throughout the adult rat in lung, testis, small intestine, adrenal, kidney , stomach, spleen heart, brain and liver (Shimasaki et al., 1991).

1.5.6.5 Actions of IGFBPs

Roles that the various IGFBPs play, viz. in prolonging the half-life of the insulin-like growth factors in the circulation, acting as a reservoir for IGF and aiding the distribution of the IGFs, have been discussed briefly above. However, in addition to these functions the IGFBPs have been shown capable of either potentiating or inhibiting the actions of IGFs. Support for these actions of IGFBPs has come exclusively from *in vitro* studies and has not been conclusively proved *in vivo*.

In inhibiting the actions of the IGFs, partially purified IGFBP-1 has been shown to reduce the incorporation of glucose into fatty acids in adipose tissue (Drop et al., 1979). Similarly, Ross et al.(1989) have shown that addition of exogenous IGFBP-1 or IGFBP-2 to chicken embryo fibroblasts, which do not produce their own IGFBP, can decrease both DNA and protein synthesis in response to administered IGFs. Furthermore, the IGFBPs have been shown to inhibit IGF receptor binding on a number of cell types in culture (Rutanen et al., 1988; Ritvos et al., 1988; Gopinath et al., 1989; Campbell et al., 1991).

Conversely, several reports have shown the potentiation of IGF action in the presence of IGFBP s under certain conditions. In studies by Elgin, Busby and Clemmons (1987), IGFBP-1 was shown to potentiate the stimulation of DNA synthesis caused in porcine aortic smooth muscle cells by IGF-I. These authors also reported a similar phenomena for IGFBP-2 in these cells. Similarly in human fibroblasts IGFBP-1 was shown to lead to an enhanced response to IGF-I alone but only in the presence of platelet-poor plasma (Clemmons et al., 1986). In another case, IGFBP-3 preincubated with human skin fibroblasts, enabled greater subsequent responses by these cells to IGF-I than when both molecules were added together (DeMellow and Baxter, 1989; Conover, 1992). Thus, under certain conditions mechanisms may exist which enable the IGFBPs to present the growth factors to the cellular receptors more advantageously.

1.6 Transgenesis

The experimental introduction of foreign DNA was first accomplished by Jaenisch and Mintz in 1974, who microinjected Simian virus 40 (SV40) into mouse blastocysts which were then re-implanted into foster mothers. In these early experiments germ-line transmission of the DNA was not achieved. Subsequently, a variety of protocols have been used to stably alter the phenotype of mice and other animals. The most common technique, that of microinjection of cloned DNA into the male pronucleus of fertilised oocytes was first made successful by Gorden et al., (1980). In addition, a second method involves the infection of early embryos with retroviruses or retroviral-based vectors bearing foreign DNA. Finally, a recently developed technique involves DNA being introduced to embryonic stem cells (ES cells) via viral transduction or transfection (Bradley et al., 1984). These stem cells are totipotent cells derived from the inner cell mass of blastocysts and can be kept undifferentiated in culture. Following transfection of the cells they can be reintroduced into a host blastocyst where they maintain the ability to differentiate *in vivo*. Thus, a transfected

ES cell when reintroduced can generate a chimaeric animal and can also contribute to the germ-line of this animal making the chimaerism heritable. A more powerful technique based on this procedure is now enabling the selective disruption or knockout of mammalian genes and subsequent generation of chimaeric mice lacking functional copies of these genes (DeChiara et al., 1991; Beddington et al., 1992).

Microinjection of cloned DNA has become the most widely and successfuly used method for transgenesis studies. In this procedure, fine glass pipettes are used to puncture the male pronucleus of a fertilised oocyte and numerous copies of the foreign DNA introduced. Typically, the transgene is incorporated into approximately 1-5 % of the eggs, as concatamers in a head-to-tail array inserted at a single locus (Palmiter and Brinster, 1986). These tandem arrays are usually inherited in Mendelian fashion as autosomal dominants (Constantini and Lacy, 1981). In rare cases the transgene can integrate into multiple sites or remain as an un-integrated episome (Lacy et al., 1983; Hammer et al., 1990; Kollias et al. ,1992). The method of chromosomal integration of transgenes following microinjection is currently unknown but models involving concatemerisation followed by non-homologous recombination have been proposed based on the similarities between integrations achieved in cell culture transfections and those achieved in transgenic animals (Bishop and Smith, 1989).

If replication of the genome occurs prior to integration of the transgene, only a subset of the cells will carry the transgene and the animal will be mosaic. Some authors have shown that up to 30 % of transgenic founder mice lines are mosaic (Wilkie et al., 1986). In these animals transmission of a transgene may not occur due to its absence in the germ cells of the founder.

Transgenesis has been used extensively to study the control of gene expression in conjunction with cell culture studies. It offers a number of advantages over cell culture. In particular transgenesis enables genes to be studied throughout the entire developmental program of the animal rather than in a single state as with cell culture. Also the gene may be simultaneously studied in every cell of the transgenic animal rather than the limited range of cell types available for *in vitro* studies. Transgenes are introduced in the absence of a selection marker, preventing prejudiced incorporation of the gene into active regions of the host cell which may influence the gene under study. Finally, the levels of expression achieved in cell culture may often be inappropriate compared to the levels seen *in vivo*. In the field of growth research, transgenesis offers advantages over infusion studies of growth

factor action by virtue of eliminating any potential immune response to the administered growth factor as well as solving many practical considerations. This may be particularly important when using growth factor analogs which may exhibit enhanced immunogenicity due to altered structure.

As well as studies examining gene regulation, transgenesis is allowing the creation of animal models for human diseases. Transgenic models for hypothalamic/pituitary disorders, sickle cell anaemias, hypertension, diabetes, muscular dystrophy and cystic fibrosis have been created (Stewart et al., 1993; Greaves et al., 1990; Lathe and Mullins, 1993). In some cases these models are enabling evaluation of gene therapy in treatment of human disease. Transgenesis is increasingly allowing the production of biopharmaceuticals. In this role, transgenes are usually targeted specifically for expression in the mammary gland epithelium and the protein product is then isolated in its native form from the milk of the transgenic animal (Bayna and Rosen, 1990). Transgenesis has also been extensively used in the fields of immunology, neurobiology and oncogenesis (see Grosveld and Kollias, 1993). Finally,with the advent of technology enabling the specific alteration or disruption of individual genes transgenesis is becoming widely used in the determination of gene function and elucidation of the developmental role played by specific genes.

1.6.1 Expression of genes in transgenic animals

The major obstacle to successful transgenesis has been attaining predictable, heritable and tissue specific expression of transgenes. Early on it was discovered that the presence of prokaryotic vector sequences in constructs could inhibit expression of certain transgenes. Although, genes including α-actin, α-fetoprotein and ß-globin were inhibited by vector sequences, others encoding immunoglobulin, elastase and collagen were unaffected (Hammer et al. ,1985; Krumlauf et al., 1985; Townes et al., 1985; Swift et al., 1985; Stacey et al., 1988; Khillan et al., 1986) suggesting that this phenomena may be dependent on the individual transgene used. Also, linear fragments with non-compatible complementary ends gave the best integration frequencies (Brinster et al., 1985). Furthermore, it has been shown that in some cases the presence of introns is required to achieve good expression (Brinster et al., 1988) (this is discussed in detail in chapter 3).

Transgenesis has been employed extensively to functionally evaluate *cis*-acting sequences responsible for tissue-specific expression gene regulation. Generally these studies involve attachment of *cis* -acting sequences from the gene under study to a reporter

genes (eg. Chloramphenicol Acetyl Transferase (CAT), ß-galactosidase, GH) and creation of transgenic animals. Such studies have enabled the regulation of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene to be fully characterised and the identification of *cis* -acting sequences responsible for its liver/kidney specific expression identified (McGrane et al., 1988,1990; Short et al., 1992). Similar studies have elucidated the promoters of genes such as elastase and gamma crystallin where tissue specific expression is controlled by a region only several hundred base pairs in length (Swift et al., 1984; Goring et al., 1987). In some cases the gene itself is used as a reporter for expression subsequent to removal of putative *cis* -acting regions, providing that its product is distinguishable from endogenous gene products (Eisenberger et al., 1992). In such cases the transgene may be 'tagged' in order to identify its product.

As the promoter regions and control of gene expression is elucidated for genes, their promoter regions become tools useful for directing the controlled expression of heterologous genes to specific tissues. Thus, regulatory regions from the mouse metallothionein (Brinster et al., 1981), rat insulin gene (Hanahan et al., 1985), mouse crystallin (Overbeek et al., 1985), rat PEPCK (Mcgrane et al., 1988), mouse ornithine decarboxylase (Halmekyto et al., 1993) and numerous other genes have been successfully used for transgenesis. However in some cases, fusion genes are expressed in novel developmental patterns due to unique combinations of promoter and heterologous gene. This was exemplified by the high brain expression observed with metallothoinein-I-rat GH transgene in mice (Evans et al. ,1985). In these animals, MT-I expression (and rGH) was never observed in the brain, indicating that the fusion gene had a unique expression profile. This expression pattern was observed in several independent lines of mice ruling out the possibility of a position effect. Also, Low et al. (1989) have identified sequences within the 3' end of the hGH gene which conferred unusual gonadotroph specific expression of a mouse MT-I-hGH fusion gene in transgenic mice. Thus, inappropriate expression of transgenes may result due to the presence within a transgene of *cis*--acting regulatory sequences whose effects are normally masked in their respective endogenous genes.

Reporter genes such as ß-galactosidase (Lac Z) have also been used to uncover novel enhancers or genes in the mouse genome. In so called 'enhancer-trap' experiments, a transgene is produced which contains a weak promoter linked to the reporter gene. This transgene is then used to generate transgenic animals randomly and the animals screened for the expression of Lac Z in interesting tissue-specific or developmental patterns. Because the

weak promoter is unable to function alone, transgene expression will only be observed where an enhancer is functional near the transgene integration site (O'Kane and Gehring, 1987). The enhancer may then be cloned by using the transgene as a probe for isolation of DNA flanking the insertion site. Alternatively, transgenes containing the Lac Z reporter gene linked to an RNA splice acceptor site can be used to identifiy interesting mouse genes. With this approach transgenes are able to be expressed if they integrate within a gene. The expression pattern of the host gene will then be reflected by the expression of the reporter (Gossler et al., 1989).

Despite the presence of promoter regions, transgenes are rarely expressed at the level of their endogenous counterparts. This is usually because the transgene does not contain all of the regulatory regions involved for expression. Also because microinjected transgenes integrate randomly into the genome, a large influence on transgene expression can often be exerted by the flanking DNA (Allen et al., 1990; Bonnerot et al., 1990). The human ß-globin genes were a good example of genes which were extremely sensitive to their position in the genome of transgenic animals and as a result were never expressed at a level comparable to endogenous ß-globin genes. However, when sequences 50-60 kb upstream of the human ß-globin genes were included in transgenes, copy-number dependent expression comparable to endogenous levels was achieved in transgenic mice (Grosveld et al., 1987; Orkin et al., 1990). This expression was achieved in several lines of mice indicating that the expression was also independent of chromosomal position. These upstream sequences were called locus control or activation regions (LCRs/ LARs) and may represent nuclear matrix attachment sites or binding sites for topoisomerase II (Townes et al., 1990). LCRs usually correlate well with the presence of DNAse hypersensitivity suggesting that their function is by way of DNA binding proteins. Such sequences have recently been identified for the chicken lysozyme gene (Bonifer et al., 1990), the α-globin gene (Higgs et al., 1990), the *fps* gene (Greer et al., 1990) and the CD2 gene (Greaves et al., 1989). Also, a putative LCR has been reported 4.8 kb upstream of the rat PEPCK gene in a hypersensitive site previously shown to be a tissue-specific enhancer (Cheyette et al., 1992; Ip et al., 1989). Whatever their mechanism of action, LCRs are able to insulate genes from the interfering presence of flanking DNA allowing correct developmental gene expression to occur. Thus, with the discovery of locus control or activation regions (LCRs/LARs) it may eventually be possible to attain copy number dependent expression of transgenes without fear of interference by flanking sequences.

The ability of transfected DNA to recombine homologously into the genome at low frequency has become the basis for a range of 'gene targeting' strategies that can generate embryonic stem cells and eventually mice in which specific genes have been disrupted or modified (Bradley et al., 1992). Gene constructs are employed which have been specially designed to undergo homologous genetic exchange with regions of the mouse genome. These vectors typically contain a positive selection marker which is transferred to the coding region of the targetted gene causing its disruption and enabling the selection of ES clones containing this alteration (Thomas et al. ,1987). The targetted ES cell lines may then be grown up individually and transferred to host blastocysts to generate individual lines of chimaeric animals. Heterozygous lines may then be bred to generate animals lacking both copies of a particular gene. More sophisticated targeting vectors should soon allow the replacement of particular genes with modified (but not disrupted) forms, providing a powerful new forum for protein structure-function studies.

1.6.2 Transgenesis, growth and the GH/IGF axis

The link between GH and animal growth was conclusively shown by the 'supermice ' experiments of Palmiter and Brinster, 1982. In these studies transgenic mice were produced carrying a mouse MT-I promoter fused to the rat growth hormone structural gene. Animals expressing the gene grew up to 1.87 times the size of non-transgenic littermates. Similar studies expressed the hGH structural gene under the control of the mouse MT-I promoter and also obtained an increased growth rate in transgenic compared to non-transgenic mice (Pamiter et al., 1983). In both studies expression was predominantly in the liver and gave rise to variable plasma levels of rGH or hGH ranging from 2-800 fold greater than the levels in non-transgenic animals. In addition, Palmiter et al., 1983 showed that the increased GH was associated with increased IGF-I levels suggesting a probable role for IGF-I in achieving the substantial increases in growth rate. Even larger transgenic mice were produced with the hGH gene under the control of the murine class I major histocompatibility (MHC) complex H-2K gene promoter. The increased growth of these animals was probably due to the ubiquitous expression of hGH in all tissues examined (Morello et al ., 1986).

Since these early studies, porcine GH (pGH) has been expressed under the control of the human metallothionein IIa promoter in transgenic mice (Vize et al., 1988). Also, bGH and hGH have been expressed under the control of the PEPCK promoter (McGrane et

al., 1988; Short et al., 1991). In both of these studies GH was found to result in increased growth of the transgenic animals produced. While transgenes under the control of the metallothionein promoters were inducible by zinc and in some cases glucocorticoid administration, the PEPCK driven transgenes were responsive to the dietary intake of the transgenic animals (Hammer et al., 1985; McGrane et al., 1988). Subsequently, the GH gene has become a useful reporter gene for studies on *cis* -acting sequences as it leads to an easily determined, non-invasive biological endpoint (increased body weight).

Over expression of a metallotheionein-rGH fusion gene was shown to correct the dwarf phenotype of *lit/lit* mice which are genetically homozygous for a chromosome 6 deficiency (Hammer et al., 1984). These mice suffer from a deficiency in GH production caused by a receptor-mediated resistance to the action of GHRF. Hence, expression of exogenous rGH was able to supplement the low levels of GH normally observed in these dwarf animals.

In contrast to studies involving overexpression of GH, transgenesis has also been used to reduce the level of endogenous GH in transgenic mice resulting in a dwarf phenotype. In these studies a pituitary-specific rat GH promoter was used to direct expression of the A-chain of diptheria toxin in the somatotroph and lactotroph precursor cells of the anterior pituitary. Any cells expressing the toxin were ablated resulting in mice lacking normal numbers of somatotrophs and hence the ability to secrete GH (Behringer et al., 1988). In a subsequent study, lines of transgenic mice were developed in which the viral thymidine kinase (*tk*) gene was driven by the rat GH promoter. Although expression of *tk* is not in itself toxic to the cells, it makes cells sensitive to the actions of certain nucleoside analog drugs, which will kill the cells. When *tk* expressing transgenic mice were treated with one such drug (1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl)-5-iodouracil or FIAU), they became dwarfed due to the selective ablation of both somatotrophs and lactotrophs (Borrelli et al., 1989).

Transgenesis has also enabled study of the relationship between GH structure and function. The importance of the third α helix of bGH for growth promotion was investigated in a line of mice containing a metallothionein-I-bGH transgene in which the bGH gene had been mutated to disrupt this helix. Mice expressing this transgene showed a significant growth supression despite the apparently unchanged receptor binding properties of the bGH analog, indicating the importance of this region for GH action (Chen et al., 1990, 1991). Additional studies in transgenic mice have shown that glycine 119 of bGH is

critical for growth promotion (Chen et al., 1991). Also transgenesis has been employed to evaluate the functions of the 22kDa, the 20 kDa and the 5 kDa N-terminal polypeptide forms of hGH. In these studies the respective coding sequences were expressed under the influence of the mouse mammary tumour virus (MTV) long terminal repeat (Stewart et al., 1992).

Aside from GH, expression of a mouse MT-I -human GHRF transgene has been shown to increase somatic growth in transgenic mice. Animals expressing the gene had measurable levels of GHRF in their serum and grew between 25-50 % larger than non-transgenic littermates (Hammer et al., 1985).

Transgenesis studies have also been performed to investigate the role of IGF-I in animal growth. Transgenic mice expressing a human IGF-I type a) cDNA linked to the mouse metallothionein-I promoter, manifested a 30 % increase in body weight and exhibited selective organomegaly of the brain, pancreas, spleen and carcass (Mathews et al., 1988). In contrast to GH transgenic mice, these animals did not exhibit enhanced skeletal growth preventing them from attaining the proportions of MT-I-GH transgenic mice. However it was noted that the circulating levels of IGF-I in the MT-I-IGF-I transgenic mice were lower than those achieved in GH transgenic mice. Therefore to characterise more fully the part played by each hormone, new strains of animals were produced from the mating of mice expressing MT-I-IGF-I with dwarf GH-deficient transgenic mice in which the GH producing cells had been ablated by expression of the diptheria toxin A chain (Behringer et al., 1990). Thus, mice generated from this cross carrying both transgenes expressed human IGF-I in the absence of GH. These animals were larger than their singly transgenic GH-deficient littermates but their growth was indistinguishable from non-transgenic mice. This study confirmed the role of IGF-I in mediating GH's stimulation of body growth including skeletal growth and suggested that while GH was necessary for the attainment of normal liver size, IGF-I could stimulate brain growth (Behringer et al., 1990). Further confirmation of IGF-I's role in brain growth has recently been attained by closer examination of the original MT-I-IGF-I animals. Despite the relatively low levels of exogenous IGF-I expressed in these mice, expression was ubiquitous throughout most tissues including the brain. Expressing mice were found to have brains which were 55 % larger than those of controls at postnatal day 55. This increase in size was due to both an increase in cell size and cell number in this organ. Furthermore, the degree of myelination observed in the

brains of transgenic IGF-I over expressors was 130 % of that in controls (Carson et al., 1993).

In confirming an essential role for IGF-II in normal embryonic growth, the technique of homologous recombination in embryonic stem cells was used to selectively disrupt one of the mouse IGF-II alleles rendering it non-functional. ES cells containing the disrupted gene were then transferred to normal blastocysts and heterozygous chimaeric animals generated in which expression of IGF-II from one allele was silenced (DeChiara et al., 1990). In this study germline transmission of the inactivated IGF-II allele from male transgenic mice resulted in offspring which were 60 % smaller than wild type littermates. However, when the disrupted gene was inherited from maternal genome the heterozygous progeny were phenotypically normal (DeChiara et al., 1991). Thus, these studies also provided evidence that the IGF-II gene is maternally imprinted such that only the male copy is expressed during normal mouse development.

1.7 Phosphoenolpyruvate Carboxykinase (PEPCK) and its promoter

The cytosolic enzyme PEPCK catalyses the conversion of oxaloacetate to phosphoenolpyruvate in the rate limiting step of mammalian gluconeogenesis (Hanson et al., 1972). This enzyme is primarily expressed in the liver/ kidney / intestine and adipose tissues, postnatally due to the change in the glucagon / insulin ratio at birth (Tilghman et al., 1976). These conditions accompanied by a rise in glucocorticoids are permissive for hepatic PEPCK synthesis. Hence, PEPCK is subject to tissue-specific developmental regulation.

The level of PEPCK in a tissue is regulated primarily by hormones, at the level of transcription (Lamers et al., 1982; Granner et al. ,1983). The PEPCK mRNA has a short half-life of approximately 30 minutes ensuring that changes in the transcription rate of the gene lead quickly to increased enzyme synthesis (Nelson et al., 1980). In some instances mRNA stability plays a part in controlling PEPCK levels (Hod et al., 1988)

The levels of the hepatic enzyme are induced by glucocorticoids (Gunn et al., 1975), cAMP (Tilghman et al., 1974) and glucagon (Inedjian et al., 1975) while they are decreased by insulin (Andreone et al., 1982). In the kidney, PEPCK is induced by changes in the acid-base balance as well as glucocorticoids but does not respond to insulin (Inedjian et al., 1975). In contrast, PEPCK expression is inhibited by glucocorticoids in adipose tissue (Neuchushtan et al. ,1988).

Cell transfection studies (transient and permanent) and transgenesis have been employed extensively to determine the *cis* -acting sequences responsible for the hormonal regulation and tissue specific expression of the rat PEPCK gene. In the transgenesis studies, mice were produced by microinjection in which varying lengths of PEPCK 5' flanking sequences were linked to either the bGH or hGH structural genes (McGrane et al., 1988, 1990; Short et al., 1992). A number of binding protein sites have been identified within approximately 500 bps 5' of the transcriptional start site (Liu et al., 1991; McGrane et al. ,1988). Subsequent mutational analysis has identified sites responsible for PEPCK's cAMP (CRE-1from -90/-82 and P3 (I) from -250/-234) (Liu et al., 1991), glucocorticoid (GRU from -455/-350) (Imai et al., 1990) and insulin (O'Brien et al. ,1990) responsiveness. Furthermore, regions required for liver/kidney-specific expression have been shown to be contained within a region from -460 to +69 bp. This region was also sufficient to confer dietary and hormonal (cAMP and glucocorticoid) responsiveness on the bGH reporter gene in transgenic mice (McGrane et al., 1990; Short et al., 1992; Eisenberger et al. ,1992). Expression in adipose tissue has been shown to require PEPCK promoter sequences between -2086 and -888 bp (Beale et al., 1991).

1.8 Aims and rationale of project

In this introduction I have attempted to review the existing knowledge on the major proteins responsible for mammalian growth. I have also attempted to provide an insight into the applications and limitations of transgenic technology . While extensive documentation exists supporting the role of IGF-I in this process, few *in vivo* studies of IGF-I action have been reported. Of those reported most were concerned with infusion of the growth factor. Our studies and others have shown that IGF-I and various analogues can restore growth or attenuate muscle degradation and nitrogen loss in rodents subject to stress (eg starvation, diabetes, gut resection,glucocorticoid treatment, hypophysectomy) (Gillespie et al., 1990; Lemmey et al., 1991; Tomas et al., 1991, 1991a, 1992, 1993). While infusion studies may be useful in addressing the endocrine actions of this growth factor they give little or no insight into relevant paracrine or autocrine actions. In contrast, studies involving production of IGF-I by transgenic animals enable the hormone to be produced locally and may be more suitable for examining these actions (Mathews et al., 1988). Also secretion of IGF-I from the cells of a transgenic animal largely mimics the normal state and may therefore allow coordinated expression of other proteins involved in the growth response, eg IGFBPs.

Furthermore, by carefully designing transgenes, delivery of the IGF-I can be targeted to specific tissues for the testing of specific hypotheses. Thus, with the long term goal of assessing the ability of IGF-I / des(1-3)IGF-I to negate the deleterious effects on growth of stressed states, my aim has been to produce a transgenic rat model expressing these growth factors under the control of the' stress responsive' PEPCK promoter region.

PEPCK is expressed only after birth, eliminating the possibility of undesirable expression of IGF-I affecting fetal development. Importantly, PEPCK is induced by conditions which normally lead to decreased production of IGF-I *in vivo* , e.g. starvation, glucocorticoid treatment, physical stress. In addition, PEPCK is predominantly expressed in the liver, the major site of postnatal IGF-I expression. Therefore the PEPCK promoter is ideally suited to stimulate endocrine release of IGF-I from the liver in situations that normally decrease IGF-I secretion. Furthermore, PEPCK is also expressed in the kidney, a tissue on which IGF-I has been shown to have stimulatory actions (Fagin et al. ,1987; Bortz et al., 1988; Martin et al. ,1991).

While mice have been routinely used for transgensis, they are less appropriate for endocrinological studies which require serial sampling of blood, surgical manipulation or metabolic measurements. In particular, mice recycle 3-methyl histidine (3-MH) , a breakdown product of actin and myosin. The urinary excretion of 3MH in rats can be used as a measure of nitrogen retention (Murray et al., 1981). Hence for the purposes of this study rats were deemed to be the experimental animal of choice.

At the commencement of this project no reports on transgenesis in rats had been published. Therefore it was important to establish the procedure in rats using a proven construct. This thesis describes the establishment of the rat transgenesis system using a metallothionein-pGH construct previously used successfully in mice and pigs (Vize et al., 1988). The growth characterisitics of these rats are also examined (Chapter 2). Secondly the construction of a PEPCK-IGF-I transgene and its expression in tissue culture cells is described (Chapter 3). After establishing that expression of IGF-I was possible with the designed construct the PEPCK-IGF-I transgene was incorporated into the germ-line of rats and its expression examined (Chapter 4).

CHAPTER 2
ESTABLISHMENT OF RAT TRANSGENESIS USING A MTIIa-pGH TRANSGENE

CHAPTER 2
ESTABLISHMENT OF RAT TRANSGENESIS USING A MTIIA-PGH TRANSGENE

2.1 INTRODUCTION:

The creation of transgenic mouse lines for use in laboratories has become routine over the past decade with numerous and varied transgenes being incorporated into the murine germ-line following microinjection (Brinster et al., 1988; Gorden and Ruddle, 1983). Although mice provide a convenient system in which to study aspects of development and expression of most genes, examination of more physiological questions may require the use of a larger animal. For example the preferred model for mammalian neurobiology and endocrinology is the rat, largely due to its physical size and hence ease of manipulation. Studies relying heavily on the use of invasive surgeries or sampling protocols too could benefit from use of a larger animal. Furthermore, in comparison to the mouse, extensive knowledge has accrued on the physiology of the rat.

In my studies the rat was chosen as the preferred species because of the eventual need to sample large amounts of blood from the experimental animals for measurement of GH, IGF-I and other blood-borne secretagogues. In addition, rats excrete 3-methylhistidine, availing us a crucial measurement of nitrogen balance in the experimental animal unlike mice which recycle this metabolite. Urinary 3-methylhistidine is derived exclusively from the breakdown of actin and myosin and therefore is a good indicator of muscle breakdown (Tomas and Ballard, 1987).

At the commencement of my candidature no literature reports had been made of the successful creation of transgenic rats. For this reason it was highly desirable for us to first attempt transgenesis in this species using a transgene construct already proven in mice. Also, it was useful to us to work with a hormone involved heavily in the growth cascade, namely GH. Not only should growth hormone be a good reporter for the transgenesis by virtue of its expected action on body growth but GH is known to be a major controller of the IGF-I axis in rats (Roberts et al., 1986). The construct chosen as a reporter for transgenesis in rats was the MTIIa-pGH transgene. This gene has been incorporated successfully into the germ-line of mice and pigs (Vize et al., 1988) and shown to express pGH in both species.

Thus, this chapter describes the production and initial characterization of transgenic rats containing the MTIIa-pGH transgene. Due to previous experience with the technique in mice, microinjection of the transgene was chosen as the preferred route of gene introduction.

All of the microinjections, embryo transfers and pGH RIAs described in this chapter were carried out by Du Zhang Tao (Ph.D student in the Department of Obstetrics and Gynaecology, University of Adelaide).

2.2 RESULTS:

2.2.1 i) About the MTIIa-pGH transgene

The construct containing the transgene was supplied to me, as illustrated in figure 2.2.1, by Vize et. al., 1988 as the PMG.01 construct. The transgene contains the efficient metallothionein II-a promoter (Karin and Richards,1982). Transcription from this promoter has been shown to be induced by both glucocorticoids and heavy metals (Karin et al ,1984a; Karin et al., 1984b). Furthermore, it has been shown to be active in a wide variety of tissues and prior to birth in animals (Andrews et al., 1991). The promoter is present as a 825 bp Hind III-Eco RI fragment (-763 to +60 of the promoter with an additional few bps of vector sequences). To the hMTIIa promoter has been abutted the pGH cDNA (522 bps) which is itself ligated to and contiguous with, a cosmid clone containing exon 5 of the pGH gene with approximately 800 bps of 3' non-translated sequence (Vize et al., 1988) (see figure 2.2.1 i)). In producing this construct a cosmid clone containing exon 5 of the pGH gene was introduced into the unique Sma I site of the pGH cDNA, replacing exon 5 of the cDNA and including sequences downstream of the last exon believed to be important for correct processing of the pGH mRNA (Woychick et al., 1982).

For transgenesis, plasmid PMG.01 was linearised with Hind III and then the 2.7 kb transgene released by redigestion with Pvu I. Preparative digests of the plasmid were electrophoresed on 1% TBE gels and the transgene fragment purified by Geneclean™ (materials and methods section 6.3.5). Digestion with both enzymes releases the MTIIa-pGH transgene with an additional 120 bp of the bacterial lac-Z gene attached to the 3' end. Figure 2.2.1 i) illustrates the structure of the transgene.

2.2.1 ii) Generation of transgenic rats

The MTIIa-pGH transgene was diluted to a concentration of 5ng/ml and 2-3 pl of DNA solution was injected into the male pronucleus of fertilized Hooded Wistar eggs. Eggs were cultured *in vitro* for 1-2 hours before being transferred into the oviducts of pseudopregnant (12-14 week old) recipients (see materials and methods section 6.3.25). Throughout this study a total of 748 embryos were microinjected, of these 546 (73 %) were deemed to be viable and were transferred following microinjection. Seventeen recipient

females were used and the transfers resulted in the birth of 70 pups. Of the pups born 39 (56 %) were found by way of either Dot-blot and/or Southern analysis (materails and methods sections 6.3.14 and6.3.15) (figure 2.2.1 ii))to possess the transgene .

2.2.2 Identification of primary transgenic rats

Tails were removed from weanling rats at 3-4 weeks of age and genomic DNA isolated as described in materials and methods (6.3.3). Dot-blots were carried out using 5 μg of tail DNA from each rat. It should be pointed out that dot-blots were carried out at different times depending upon the arrival of new litters. Thus it does not seem informative to include every blot performed. Only blots which illustrate animals featured throughout this study have been included. The hybridisation probe used for analysis was the 540 bp Hind III-Ava I fragment from the 5' end of the MTIIa promoter. This probe has been successfully used to identify mice and pigs carrying the MTIIa-pGH transgene (Vize et al., 1988). Following an initial round of microinjection and embryo transfer, a number of animals were identified as possessing the transgene by Dot-Blot analysis. Figure 2.2.2 i) illustrates the identification of several animals including MT-1(first expressing transgenic)(column 2, row b), A (column 7, row d) and C (column 6, row d), found to possess the transgene. Following scintillation counting of the radioactivity in each dot, these animals were found to have 80, 8, and 1 copies of the transgene respectively when compared to the copy number control dots. It should be noted that several other animals on this blot have also integrated the transgene but will not be discussed. The high background hybridisation present even in the negative control (non-transgenic rat DNA) has been a constant problem in analysis of these animals, making identification of low copy animals virtually impossible. Background hybridisation has also been observed in genomic Southern analysis of rats throughout the study. This background most likely represents cross hybridisation to the rat metallothioinein IIa gene(s). Such background has meant that selection of animals for breeding relied upon additional criteria, .eg. production of pGH in the blood of the rats.

Figure 2.2.2.ii) shows the integration of the transgene into 2 rats, B(column 9, row d) and D (column 1, row c) from 3 further litters. Again integration of the transgene can be seen to have occurred in a very high percentage of the animals featured. Animal MT-1 has been included on this blot as a positive control (column 2, row b). Comparison of the two blots illustrates the inter-experiment variation associated with performing such analysis on

different occasions. It can be seen that the dilution series used as a copy number control in both blots is giving quantitatively different signals making absolute copy number determination difficult. For my purposes such blots served as an initial rapid screen in combination with the pGH radioimmunoassay. Where possible high copy number animals were studied due to ease of identification.

A later series of microinjections and embryo transfers led to the production of 32 animals of which 12 possessed the transgene in low copy number. Seven of these animals were selected for breeding on the basis of pGH expression.

2.2.3 Integration of the MTIIa-pGH transgene

In order to examine the organisation of the integrated transgenes in the rats, Southern analysis was performed using either Eco RI or Bam HI. As there are no Bam HI sites within the transgene itself digestion of genomic DNA from transgenic rats with this enzyme should yield a single MTIIa promoter hybridising band for each site of integration. The size of the fragments will depend upon the distance from the site of integration to the nearest Bam HI site. Figure 2.2.3 i) shows Bam HI Southern analysis of several primary MTIIa-pGH transgenic rats. Rats MT-1, D and M5.5 (lanes 3, 5 and 6 respectively) exhibit a single band of high molecular weight hybridisation, indicated with an arrowhead, due to integration of the transgene at a single site within the genome. Although of apparently the same molecular weight on this gel, resolution of bands of this large size could only be achieved on a lower percentage agarose gel. Upon prolonged autoradiography of this filter a single integration site was observed for the low copy number animal C. Even after long exposure no corresponding bands were seen in the control lane verifying that the hybridisation being seen in lanes 3, 5 and 6 is due to the transgene. Digestion of genomic DNA with EcoRI, an enzyme which cuts the transgene in two places can be expected to yield information on the organisation of individual integration events. Figure 2.2.3 ii) shows an Eco RI Southern analysis performed on 20 μgs of tail genomic DNA from rats MT-1(lane 5), A (lane 2), B (lane 3) and C (lane 1). All four rats exhibit three common bands of approximately 6 kb, 2 kb and 1.2 kb. These species are also present in the DNA of another MTIIa-pGH microinjected rat shown in lane 7, but are absent from the DNA of a non-transgenic animal (lane 6). In addition rats MT-1, A and C possess a band of approximately 0.9 kb. (although barely visible in C), while rat B exhibits a smaller 0.3 kb species. Later Southern analysis on A (data not shown) indicated that the 1.2 kb band may be due to incomplete digestion of

the DNA. The 0.9 kb band present in MT-1, A and C is diagnostic of head-tail integration of the transgene in these animals (see figure 2.2.3 iii)) and therefore suggests that rat C has at least one and a half copies of the transgene integrated. The relative intensities of this band vary in proportion to the number of gene copies integrated. The presence of the higher species are not easily explicable as their size does not correspond to either head-head or head-tail organisation of the transgenes, but perhaps arise due to Eco RI sites flanking the sites of insertion. However, it is unusual that these fragments would be of identical size in each individual animal given the assumption of random transgene integration. It is possible that at least some of these common species represent the previously mentioned cross hybridisation of the MTIIa probe with endogenous rat metallothionein genes. The small molecular weight species present in B may be due to rearrangement of the MTIIa-pGH transgene at the site of integration leading to a deletion of part of the transgene.

2.2.4 Blood-borne pGH and growth of primary MTIIa-pGH rats

At the time of weaning when tail was removed for DNA analysis, a small amount of blood was sampled from each animal. This blood was subject to radioimmunoassay for pGH. In four animals, blood taken at the time of sacrifice was assayed for IGF-I. Animals were also weighed from the time of weaning at weekly intervals. Table 2.2.4 lists the plasma levels of immunoreactive pGH present and IGF-I in the blood of transgenic rats as well as the relative growth of each animal compared to sex-matched non-transgenic littermates at 12 weeks of age. As can be seen levels of plasma pGH varied significantly between individual animals. No correlation was observed between the transgene copy number of these animals and their pGH levels, for example rat C is a low copy number animal and yet produced more pGH than rat D, who has many more gene copies. In general female animals produced higher levels of pGH and reached a larger size at weaning relative to their controls than did males. At 12 weeks of age male expressors were slightly larger than females of the same age consistent with the sexual dimorphism reported for rat growth (Jansen et al., 1983). A correlation was found between the circulating levels of pGH in females and their body size at 12 weeks of age ($r=0.943$, $P<0.01$). However, there was no such relationship between the growth attained in all rats and their plasma level of pGH, although the two animals which attained the highest relative size were the highest pGH producers. In several animals (D and M3.4) essentially normal growth was maintained even in the presence of pGH. Figures 2.2.4 i), ii) and iii) illustrate the growth profiles of several pGH producing rats from

weaning. These curves show that the dynamics of growth for each transgenic rat initially produced were somewhat variable. Most pGH producers began to outgrow littermates from weaning onwards. However, it is possible that growth was manifested prior to this point, since a number of animals were much larger from birth than their littermates. Studies are underway to map more precisely the growth of transgenic rats prior to weaning. Figure 2.2.4 iv) and v) show the much greater body size of two pGH expressing transgenic rats than their respective sex matched littermates at 13 weeks of age. Female rat C was almost twice the size of her non-transgenic littermate at this stage. Thus the animals produced can be divided roughly into two groups, those which exhibit enhanced growth (generally have high plasma pGH) and those which grow essentially normally despite the presence of pGH.

2.2.5 Breeding of primary MTIIa-pGH rats and inheritance of the transgene

Throughout the study attempts were made to breed most of the primary pGH producing transgenic rats. In some instances animals died before mating was successful . We were only able to successfully breed 4 of the primary rats (A, C, D and M5.5), three of which were female and one male. The F1 generation animals produced were assayed for the inheritance of the transgene by dot-blot and/or Southern analysis shortly after weaning. As with the founder animals the offspring were weighed at weekly intervals and plasma pGH levels measured.

Animal A sired two litters of offspring (rows a and b). From dot-blot analysis (figure 2.2.5 i)) 3 males and 2 females appear to have inherited the transgene in a similar copy number to their parent (26 %), whereas as many as five others may have inherited lower numbers of copies of the transgene. Preliminary Southern analysis of these F1 animals suggested that none had inherited the head-to -tail arrangement of the transgene from the parent (data not shown). The single litter produced from mating of rat C contained 13 animals (rows c and d). In this case because of the low copy number of the founder animal, the dot-blot does not easily discern which animals inherited the transgene. It does however show that one male animal inherited more copies of the transgene than the parent (column 2, row c). This could be explained if founder C was a mosaic animal in which there was an uneven distribution of transgene copies within cells of the germ-line and the somatic cells. However, as DNA loadings on this blot have not been normalised relative to an endogenous single copy murine gene, small differences in DNA loadings may account for this result. Founder D gave birth to a single litter of 12 pups, 7 of which were male and 5 female (row

e and column 12, row d). Dot -blot analysis showed that 4 male and 2 female (50%) inherited the transgene in similar copy number to the parent. Southern analysis with Eco RI on animals from this litter (figure 2.2.5 ii)) demonstrates that all of the animals identified as positive by dot-blot appear to have the 0.9 kb band (indicative of head to tail transgene integration), except male D1.4. and D1.10. In the case of D1.10 the genomic DNA used in this Southern blot was seen to be incompletely digested. Hence this may explain the absence of a hybridising 0.9 kb band in lane 7. In addition to the 0.9 kb fragment in lanes 2, 3, 5, and 8, a fainter 1.6 kb species can be seen these tracks. This fragment corresponds with the size expected for head-head copies of the transgene. Also a higher fragment of approximately 2.0 kb can be seen in several F1 offspring (lanes 3, 5, 6 and 8). This fragment is absent from the digest of founder D's DNA but a slightly higher band of approximately 2.5 kb is present. These bands may represent junction fragments between the transgene integration site and the Eco RI sites flanking the insertion site. If this is the case then a rearrangement of the transgene organisation may have taken place in the F1 offspring to generate a smaller 2.0 kb junction fragment. Such rearrangements have previously been reported for the germ-line transmission of a foreign β-globin transgene in mice (Lacy et al., 1983).

The higher molecular weight bands in the autoradiograph are present in the DNA of the non-transgenic control animal (lane 1) and therefore are likely to represent cross-hybridisation of the hMTIIa probe with endogenous rat metallothionein gene(s).

Breeding of an additional pGH producing animal, M5.5 gave rise to a litter of 8 pups only one of which was shown by Southern analysis (figure 2.2.5 iii)) to have inherited the transgene. The low frequency of inheritance in this line along with the variation in copy number amongst F1 animals produced from all breedings, points to the possibility that a large number of the primary transgenic rats may have been mosaic. It has been observed that up to 30 % of transgenic founder mice produced by microinjection are mosaic (Wilkie et al., 1986). In addition,because of lack of sufficient genomic DNA, I cannot exclude the possibility of integration of the transgene into several chromosomal sites in Founder A.

As pointed out earlier, pGH assays were carried out on the plasma of the F1 offspring and the animals weighed weekly (see Table 2.2.5.). In line with Southern analysis of the F1 progeny of rat A (data not shown), none of these animals was found to be producing pGH. Hence, it seems possible that the functional copy/copies (present as head-

to-tail multimers) of the transgene have been lost in these animals. Only a single female animal from breeding of C was found to be expressing pGH. This animal had a very similar level of pGH (717 ng/ml compared with 800 ng/ml) to its transgenic parent and exhibited a similar enhanced rate of growth. F1 animals from breeding of D all possessed low levels of blood-borne pGH (between 13-25ng/ml) and grew at a similar rate to their non-transgenic littermates, as had their transgenic parent. Breeding of M5.5 produced a single female animal which inherited its transgenic mothers enhanced rate of growth but had a somewhat lower level of plasma pGH (25 ng/ml compared with 42 ng/ml).

A number of expressing F1 offspring were bred with non-transgenic animals to produce an F2 generation and examine the stability of the transgene through another generation. C1.13 produced a litter of 13 pups, none of which was shown to be expressing pGH in their blood. Rat D1.6 sired 3 litters (a total of 19 offspring), 42 % of which were found to have the transgene by Southern analysis (see figure 2.2.5.iv)) or by Polymerase Chain Reaction (PCR) (data not shown), using primers specific for the hMTIIa promoter (see materials and methods section 6.3.13). In figure 2.2.5 iv) both a 0.9 kb and 1.6 kb fragment, indicating head-tail and head-head integration of the transgene respectively are seen in lane 1 (D1.6). These fragments are also observed in lanes 4, 5,6,8,11, 13 and 14 indicating inheritance of the same transgene organisation by the F2 animals. A 2.4 kb and 1.3 kb fragment are also observed in all lanes, most likely due to cross-hybridisation of the probe with endogenous rat metallothionein gene(s). These animals also, possessed low levels of pGH and exhibited normal growth compared with non-transgenic littermates. Mating of M5.5.4 produced an F2 generation of 6 animals. PCR of tail genomic DNA (figure 2.2.5 v)) was used to show that 4/6 had inherited the transgene. These animals had 49, 46, 37 and 68 ng/ml of pGH in their plasma respectively at the time of weaning and all were seen to grow at an enhanced rate relative to non-transgenic littermates. Hence it appears that the MTIIa-pGH transgene has been stably inherited into the F2 generation in two separate lines of transgenic rats. Figure 2.2.5 vi), illustrates the entire family trees of rat M5.5 (high expressor/fast growing) and rat D (low expressor/normal growth)

2.2.6 Expression of the MTIIa-pGH transgene

Where possible at death, tissues were taken from pGH expressing transgenic rats and RNA extractions were carried out. Subsequently 20 or 50 µgs of total RNA was subject to analysis by ribonuclease protection assay (RPA) (see materials and methods section

6.3.17). The antisense probe used in this analysis was generated by T7 transcription and corresponded to approximately 120 base pairs at the 3' end of the porcine growth hormone gene, cloned into pSP72. T7 transcription of the Pvu II linearised insert gave a 150 bp riboprobe. The 150 bp probe (containing polylinker sequence from its vector host), protected a band of approximately 120 bp in an assay containing pig pituitary mRNA, which served as a positive control (see figure 2.2.6 ii)). In some assays this probe protected a number of species in RNA from non-transgenic rats but did not protect the full 120 bp RNA expected in pGH expressing animals.

Initially, a wide variety of tissues (liver, kidney, intestine, brain, leg muscle and heart) from three individual pGH expressing animals (MT-1,C1.13 and D) were analysed for pGH mRNA (see figures 2.2.6.i) and ii)). These animals each possessed different levels of blood-borne pGH protein. A similar distribution of the pGH mRNA was observed in two of these animals (MT-1 and C1.13), with the highest levels observed in the livers of both and the intestine being the second highest expression site. The signal present in other tissues was extremely low and therefore difficult to quantify. However it appeared that there were differences in the preferential sites of expression amongst the other tissues, with the pattern as follows:

MT-1: Kidney>muscle>heart>brain

C1.13: Intestine=Lung>brain>kidney

The third animal (D) in this group exhibited striking differences in the transgene expression pattern (see figure 2.2.6 ii)). In this animal the major site of expression was the intestine (confirmed in three independent protection assays). followed by the lung, liver, kidney and brain. Lung and brain data are not shown here.

A further four expressors (M4.7,M4.4,M1.9 and M2.1) have been analysed for the production of the pGH mRNA (figure 2.2.6.iii) and iv)) With these animals only liver, kidney, intestine and spleen were assayed. As spleen was found to be expressing pGH mRNA in transgenic mice produced from this construct within our department (Mrs.L. Crocker, personal communication), it was included for analysis. In all four animals the major site of expression was once again found to be the liver. M 2.1 had the highest levels of pGH expression out of all animals examined, expressing equally well in the intestine and the liver (figure 2.2.6 iv), lanes 4 and 6). This animal also expressed the pGH message highly in kidney and spleen (lanes 5 and 3). Amongst the remaining three animals (figure 2.2.6 iii)) expression was a much lower in the remaining tissues, with a tendency towards

kidney>intestine>>spleen.

With the finding that expression was highest in the intestine of animal D, it was of interest to determine whether this expression pattern had been inherited by other animals in this family. Figure 2.2.6 v) shows RNAse protection analysis of offspring within the D family. This figure shows that founder D exhibited much higher expression than any of the F2 progeny of D1.6. Although their expression is an order of magnitude lower (50 µgs of each RNA has been used), animals D1.6.4 and D1.6 12 show the same preferential intestinal expression as D. Furthermore this result has been observed in three independent assays.

In summary, the major site of expression in most animals was the liver with high expression also observed in the intestine of three animals from the same family. It appears that all animals also express lower levels of the pGH mRNA in various other tissues including the lung, kidney and spleen. This is consistent with the housekeeping role of the metallothionein promoter in its corresponding native gene.

2.2.7 Zinc induction of the MTIIa-pGH transgene

As the MTIIa promoter contains metal-responsive elements (Karin and Richards, 1987) it was decided to test whether the low level expressors (D family) were inducible by zinc. In order to test this hypothesis, 9/12 F1 animals were placed on a diet in which Zn^{++} (25 mM) was included in the animals' drinking water for a period of 2 weeks. Blood was sampled from these animals prior to commencement of the treatment, following the treatment and subsequent to the removal of the Zinc. RIA of the plasma samples revealed that 3/9 of these animals were inducible in the presence of Zn^{++} (see table 2.2.7)). The level of induction ranged from 39%-95%. Subsequently, two F1 animals have been bred and their offspring are currently being subjected to a similar experiment. Also a similar experiment has been performed on the F1 generation of M 5.5 (data not shown) and showed that there was no change in the plasma pGH level of M5.5.4, the sole transgenic in this litter. More detailed experiments are being planned to ascertain the optimal levels of zinc required for this induction in an effort to better understand the basis of such a phenomenon in the D family of transgenic rats.

It would also be interesting to examine the response of plasma pGH to nutritional or physical stress in these animals due to the known glucocorticoid inducibility of the MTIIa promoter (Karin et al., 1984).

2.2.8 IGF and IGFBPs in pGH expressing rats

Growth Hormone is a known regulator of both IGF-I and the IGFBPs especially IGFBP-3 (Zapf et al.,1989). For this reason it was of interest to examine the production of IGFBPs in the plasma of the pGH expressing transgenic rats and in addition look at the effect of GH expression on endogenous IGF-I expression.

Blood samples were taken from several animals at death and Western ligand blots (materials and methods section 6.3.23) carried out on diluted plasma. Figure 2.2.8 i) shows a ligand blot probed with ^{125}I-IGF-II tracer. The blot shows the existence of six clearly defined species. A triplet of proteins (40-50 kDa.) followed by a doublet (28-30 kDa.) and then a lower single protein (24-25 kDa.). This pattern has been previously observed for normal rat plasma (Luo and Murphy, 1990) and these proteins are thought to correspond to IGFBP-3, IGFBP-1 and IGFBP-2 respectively. However,to categorically define these ligand blotted species, purified sources of these BPs would need to be run in parallel on the same gel and immunoblots performed using the appropriate antisera. Although the pattern of IGFBPs is the same between the individual transgenic animals shown here, the intensities of different binding proteins varies. Unfortunately these animals were not killed at the same age making any slight developmental differences in BP production difficult to rule out. It should be noted however, that all animals had reached sexual maturity. No consistent increase in one particular BP species appears to occur in all expressors. Animals D, M1.9 and M2.1 show a definite increase in the higher molecular weight triplet (40-50 kDa.) generally associated with rat IGFBP-3 (Yang et al., 1989). Furthermore, animals C1.13, D and M2.1 have an increase in the approximately 29 kDa protein. Only animal C1.13 shows any increase above control of the lowest molecular weight protein (24-25 kDa.). Staining of a duplicate gel run concurrently, using Coomasie blue suggested that these fluctuations were not due to loading differences between samples.

In addition to Western ligand blots, Northern analysis was carried out on the liver RNA of several expressing transgenic rats. Northerns were probed initially with rat IGF-1 then rat IGFBP-3 and IGFBP-1 sequences. Subsequently filters were probed with a rat GHBP/receptor probe, which contains extracellular domain sequences common to both the GH receptor and the circulating GH BP (Tiong and Herington, 1991). Finally filters were probed with an 18S ribosomal RNA probe to examine RNA loadings in each lane.

Figure 2.2.8 ii) a) shows the expression of rat IGF-1 in several animals. Expression was categorised by a large number of different sized messages, grouping broadly into three major species of 0.8 kb, 1-2 kb and 7-8 kb. Only animals C1.13 and M2.1 are expressing all three groups of messages. The control animal and other expressors appear to produce only the lower molecular weight species. Densitometric scanning and quantitation of the 0.8 kb species confirmed the significant increase in the abundance of this message in animals C1.13 and M2.1 relative to the non-transgenic control. Such increases in the abundance of IGF-1 mRNA species have previously been observed in hyphophysectomised rats injected with GH (Roberts et al, 1986), *lit/lit* mice (Mathews et al., 1986) and in GH transgenic mice (Mathews et al., 1988). When this filter was reprobed with a rat IGFBP-3 probe no signal was obtained even after low stringency washing conditions. The reason for this result is not known, although it is possible that expression of IGFBP-3 is too low to be readily detected by Northern analysis in adult rats. In support of this suggestion, Albiston and Herington (1992) have used a more sensetive S1 nuclease protection to detect IGFBP-3 mRNA in rat tissues. The filter was then stripped and reprobed with IGFBP-1 (figure 2.2.8 ii) b)), only animals C1.13 and M 2.1 were seen to be expressing the 1.6 kb mRNA species This apparent induction of IGFBP-1 mRNA is contradictory to the down regulation of this mRNA species observed upon injection of GH to hypophysectomised rats (Seneviratne et al.1990). In contrast to the IGF-I results the levels of IGFBP-1 expression were greater in rat C1.13 than in M2.1. A higher 2.5-3 kb species of mRNA can be seen in RNA from C1.13. Such higher molecular weight messages have also been previously reported in the GH-treated hypophysectomised rat (Seneviratne et el., 1990).

When expression of the rat GH BP/Rreceptor was examined (figure 2.2.8 ii) c)), several different messages were seen , with the two predominant ones being 1-2 kb and 4-5 kb respectively. These mRNA species have been shown to correspond to the GH BP (1-2 kb) and GH receptor (4-5 kb) (Tiong and Herington, 1991). When densitometry was carried out on the lower molecular weight species, again rats M.2.1 and C1.13 were found to have greatly elevated levels of this mRNA compared to all other individuals. However expression of the GHBP was also elevated in D and to a minimal extent in M1.9. Similar increases in liver GHBP mRNA have been seen following continuous infusion of GH to female hypophysectomised rats (Maiter et al., 1992).

Figure 2.2.8 ii) d) shows probing of the same filter with an 18s rRNA probe for determination of RNA loadings. Although RNA loadings can be seen to vary across the

filter, all increases in mRNAs discussed above are real when normalised to the loadings of RNA in each track. Figure 2.2.8 iii) shows the densitometric quantitation of the 0.8 kb IGF-1 mRNA and the GHBP mRNA normalised to the RNA loading in each lane of the filter.

FIGURE 2.2.1 i)

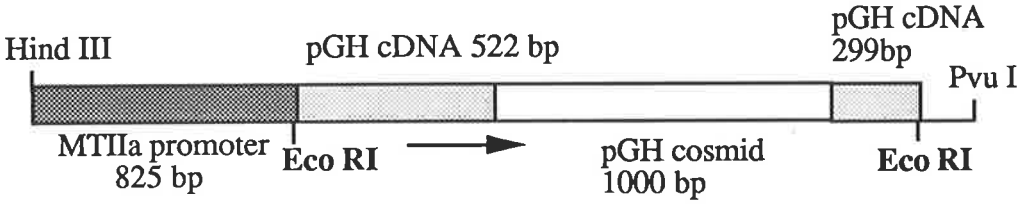
A linearized representation of the 2.7 kb Hind III-Pvu I MTIIa-pGH transgene.

FIGURE 2.2.1 ii)

Shows the overall results of transgenesis in rats using the MTIIa-pGH construct.

FIGURE 2.2.1 **Structure of the 2.7kb MTIIa-pGH transgene**

i)



ii) **Production of hMTIIa-pGH transgenic rats**

No. embryos used	No. embryos microinjected	No. embryos transferred	No. embryo recipients	No. pups born	pups with transgene
923	748	546	17	70	39

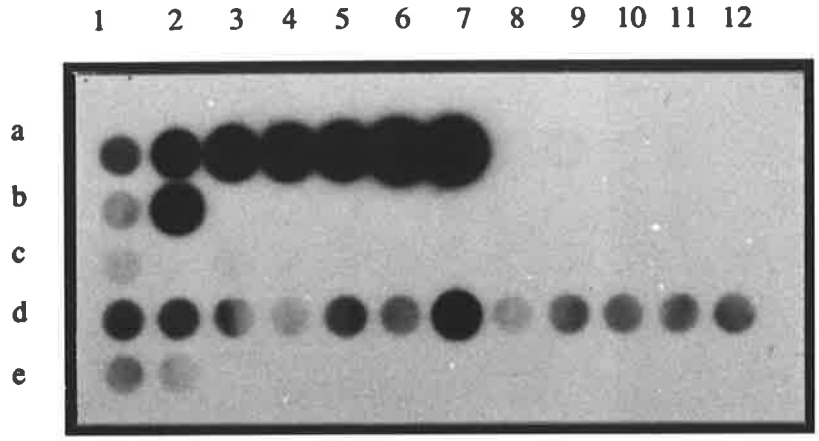
FIGURES 2.2.2 i) and **2.2.2 ii)** Illustrate the identification of several primary transgenic rats by dot-blot using the MTIIa promoter Hind III-Ava I probe. 5ug samples of DNA isolated from rat tails at weaning, were denatured and applied to a Nytran membrane along with a negative control sample fom a non-transgenic rat. Also a series of samples with increasing amounts of the transgene containing plasmid were applied after being mixed with 5ugs of the negative control DNA. The amounts of plasmid used corresponded to gene copy numbers of between 1 and 160 copies per cell. This was calculated by taking into account the proportion of the plasmid homologous to the hybridisation probe (approximately one fifth) and assuming a rat diploid genome size of 5 pg (Sober et al., 1970). In row **a** the number of transgene copies per cell, is given with the number in brackets indicating the equivalent pg amount of plasmid DNA applied to the filter. Filters were hybridised overnight at 42°C and were washed up to 42° C in 0.1 x SSC /0.1 % SDS for 30 minutes before being autoradiographed overnight. A key illustrating the identity of each sample is presented below for i) and ii). Where only a date is given, all animals on that row indicated with a dash, were from a litter born on that date.

i)	1	2	3	4	5	6	7	8	9	10	11	12
a	12.5 (1)	100 (8)	300 (24)	400 (32)	500 (40)	1000 (80)	2000 (160)					
b	neg.	MT-1										
c	b 8/3	-										
d	b20/3	-	-	-	-	C	A	-	-	-	-	-
e	b26/3	-										

ii)	1	2	3	4	5	6	7	8	9	10	11	12
a	12.5 (1)	50 (4)	100 (8)	200(16)	400 (32)	500(40)	1000 (80)					
b	neg.	MT-1										
c	D b28/4	-	-	-	-	-						
d	b5/6	-	-	-	-	-		b18/6	B	-		

Dot-Blot Identification of MTIIa-pGH Transgenic Rats

i)



ii)

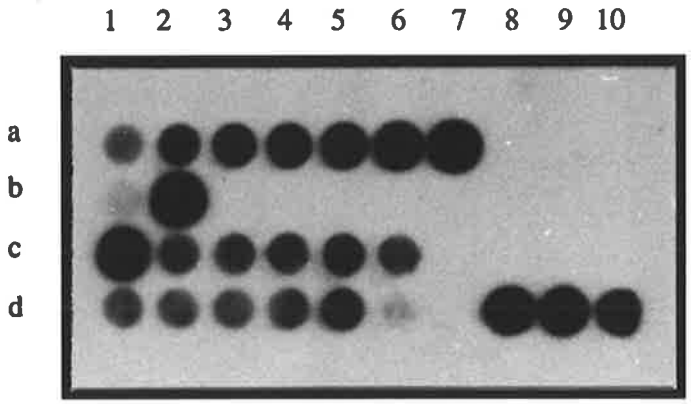


FIGURE 2.2.3 i) Twenty micrograms of rat genomic DNA was digested with Bam HI after spermine precipitation. Subsequently samples were electrophoresed on a 0.8 % TBE Agarose gel. The gel was transferred to a Nytran filter and was probed with the oligolabelled Hind III-Ava I MTIIa promoter. The filter was washed at high stringency for 1hour at 65°C in 0.1 x SSC/0.1 % SDS before being autoradiographed overnight. The sizes of radiolabelled SPP1 molecular weight markers run on the same gel (lane 1) are given on the left of the gel. The other lanes were loaded as described below.

lane 2: non-transgenic rat

lane 3: rat MT-1

lane 4: rat C

lane 5: rat D

lane 6: rat M5.5

The arrowhead on the right of the gel indicates the single integration site for the transgene in each of the animals analysed.

FIGURE 2.2.3 ii) Rat genomic DNA (20 µg) was digested with Eco RI and electrophoresed on a 0.8 % TBE Agarose gel before being transferred to a Nytran membrane and being probed with the MTIIa ,Hind III-Ava I fragment. The size of SPP1 molecular weight markers electrophoresed on the same gel are given on the right hand side of the autoradiograph.

Lane 1: rat C

Lane 2: rat A

Lane 3: rat B

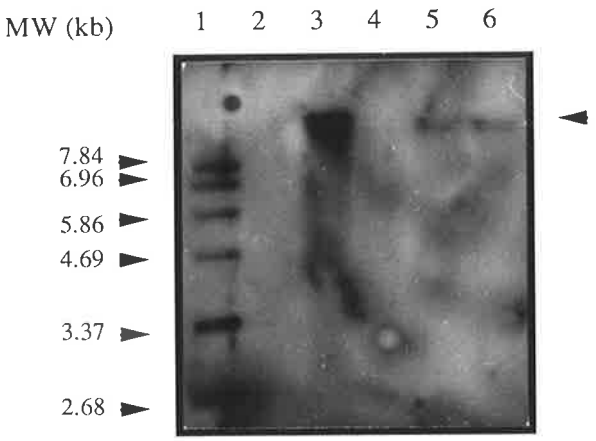
Lane 4: rat born 4/6/91

Lane 5: rat MT-1

Lane 6: non-transgenic control rat

Lane 7: rat born 8/3/91

Bam HI Southern Analysis of MTIIa-pGH Rats



Eco RI Southern Analysis of MTIIa-pGH Rats

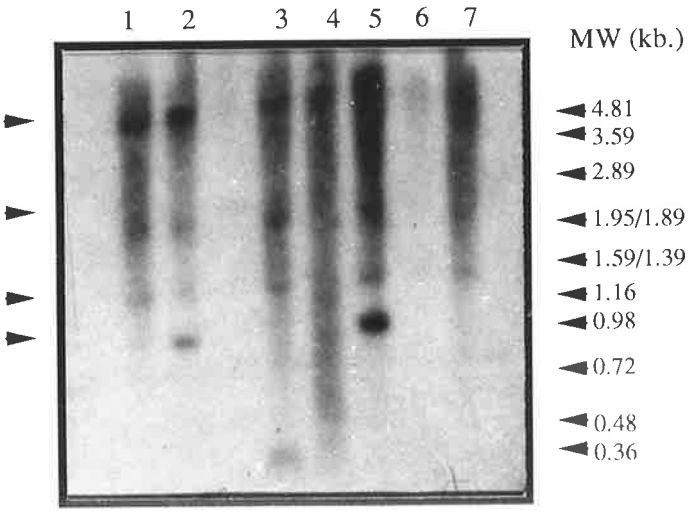
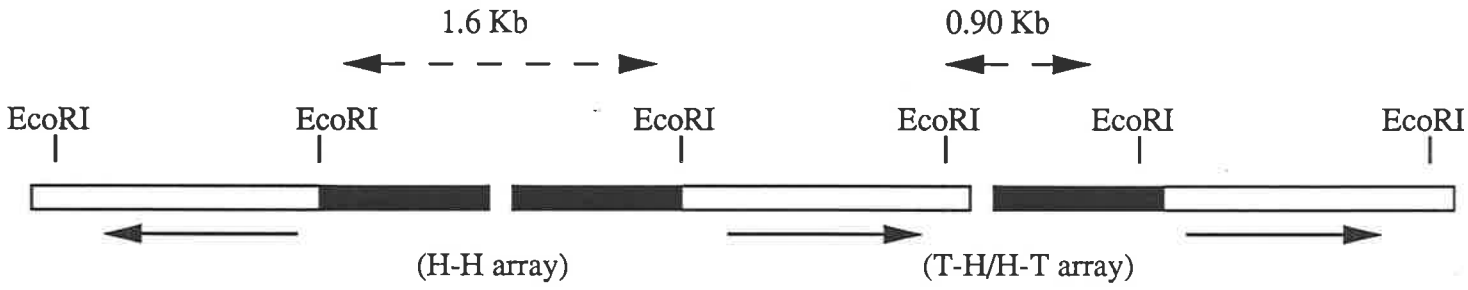


FIGURE 2.2.3 iii) **INTEGRATION AND POSSIBLE ORGANISTION OF MTIIa-pGH TRANSGENES IN TRANSGENIC RATS**



= MTIIa promoter fragment

= pGH cDNA +transcriptional terminator

= direction of transcription

Characteristics of Primary MTIIa-pGH rats

ID.NO.	SEX	serum pGH (ng/ml)	serum IGF-1 (ng/ml)	Relative size Weight ratio
A	M	64	n.d.	1.24
B	M	9	n.d.	1.00
C	F	800	n.d.	1.81
D	F	18	942	1.2
M1.2	M	85	n.d.	1.28
M1.9	F	1192	1146	2.0
M2.1	F	1170	1074	1.97
M3.4	F	23	n.d.	0.96
M4.4	M	360	574	1.41
M4.7	F	712	n.d.	1.78
M5.2	M	529	n.d.	1.25
M5.5	F	42	n.d.	1.72

TABLE 2.2.4

This table shows the sex, serum pGH and relative size ratio of the primary MTIIa-pGH rats. The weight ratio was calculated as the live weight of each transgenic animal divided by the mean live weight of sex-matched non-transgenic littermates at 12 weeks of age. There were no other females in the litter in which rat D was born. Her relative growth rate is expressed as her weight at 12 weeks of age divided by the average weight of her three non-transgenic F1 daughters. Serum pGH and IGF-1 were measured using specific radioimmunoassays (Materials and methods section 2.3.22 and Owens et al., 1990).n.d.=not determined. (Non-transgenic rats assayed for IGF-I had 550-600 ng/ml IGF-I)

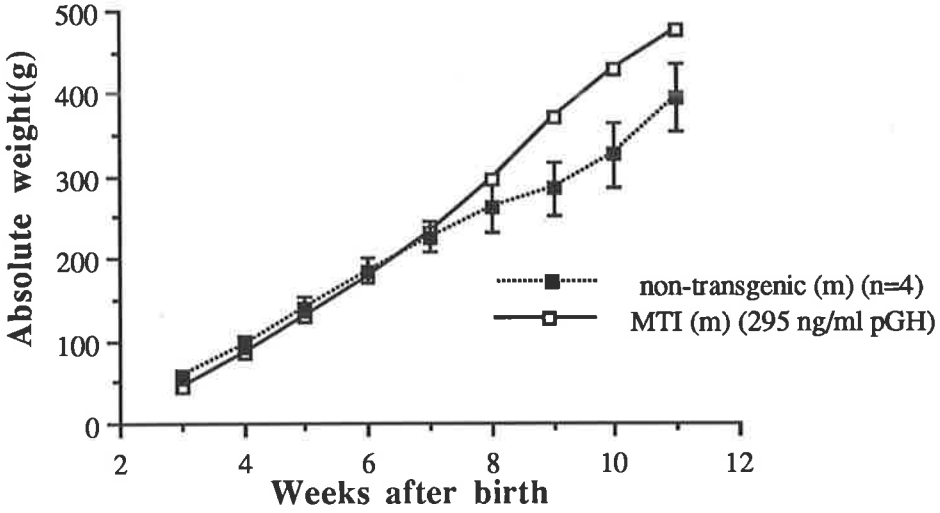
FIGURE 2.2.4

i) Shows the growth curve of the intial pGH expressing male rat MT-1. This animal was expressing 295 ng/ml of pGH and as the graph shows began to outgrow his 5 male (m) littermates approximately seven weeks after birth.

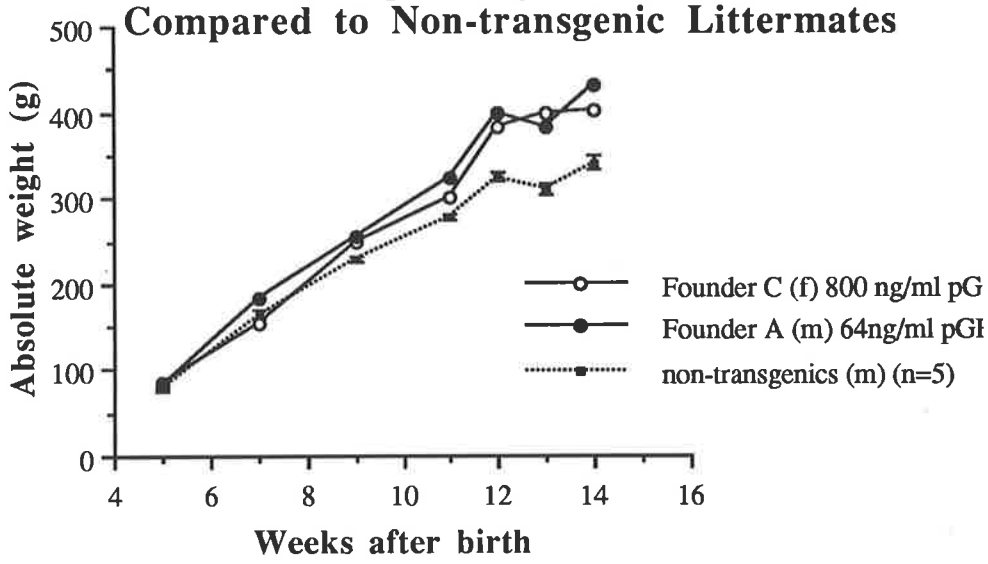
ii) Shows the growth curves of two expressing MTIIa-pGH rats, A and C compared to the growth of male non-transgenic littermates. Even though C is female (f) she has still outgrown the males of this litter.

iii) Shows the growth curves of five female pGH expressing rats compared to their non-transgenic littermates. In these animals the increased growth can be seen after weaning. Interestingly, animal M3.4 exhibits essentially normal growth even in the presence of 23 ng/ml pGH. NT= non-transgenic.

Growth of First Expressing MTIIa-pGH Compared To Non-transgenic Littermates



Growth of Expressing Founder Rats Compared to Non-transgenic Littermates



Growth of Expressing pGH Rats Compared to Non-transgenic Littermates

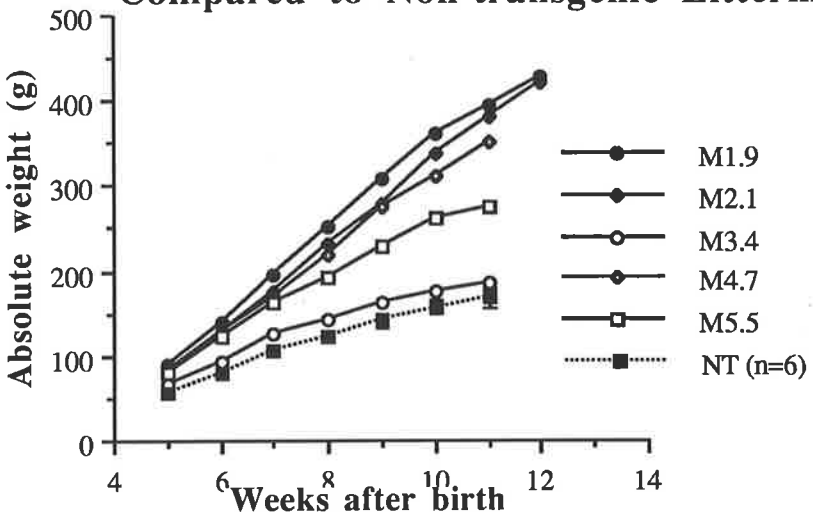


FIGURE 2.2.4

iv) A photograph of male A (64 ng/ml pGH)(above) compared with its non-transgenic littermate (below) at 13 weeks of age.

v) A photograph of female C (800 ng/ml pGH)(below) compared with its non-transgenic littermate(above) at 13 weeks of age.

FIGURE 2.2.5 i) Dot-blot analysis was carried out on 5 µgs of genomic tail DNA from the F1 generation of founders A,C, and D. DNA was denatured and transferred to a Nytran membrane. The probe was the oligolabelled Hind III-Ava I fragment of the MTIIa promoter. The filter was washed up to 42°C for 1 hour in 0.1 x SSC/ 0.1 % SDS before autoradiography overnight. DNA from the founder animal of each litter has been included on the filter as a control. The grid below is a key to the samples on the filter

	1	2	3	4	5	6	7	8	9	10	11	12
a	A	A1.1	A1.2	A1.3	A1.4	1.5	1.6	1.7	1.8	1.9		
b	A2.1	A2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	2.10		
c	C	C.1.1	C1.2	C1.3	C1.4	C1.5	C1.6	C1.7	C1.8	C1.9	C1.10	C1.11
d	C1.12	C1.13										D1.12
e	D	D1.1	D1.2	D1.3	D.14	D1.5	D1.6	D1.7	D1.8	D1.9	D1.10	D1.11

Inheritance of the MTIIa-pGH Transgene

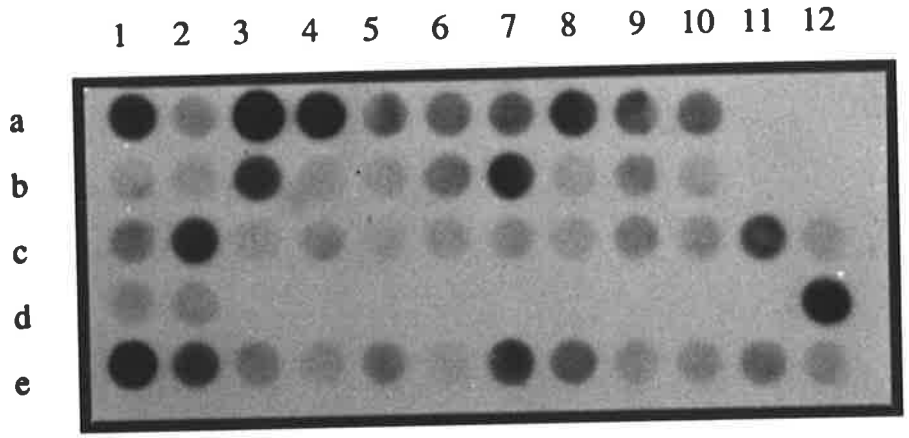


FIGURE 2.2.5 ii) 20 µgs of tail genomic DNA was digested to completion with Eco RI (except in the case of animal D1.10 where digestion was seen to be incomplete following electrophoresis). Samples were electrophoresed on a 0.8 % TBE Agarose gel before being hydrolysed, denatured neutralised and transferred to Nytran membrane. DNA was crosslinked to the filter and the filter hybridised in the presence of the Hind III-Ava I MTIIa promoter probe. The filter was subsequently washed up to 42°C for 30 minutes in 0.1 x SSC/0.1 % SDS and autoradiography carried out for 72 hours. The sizes of molecular weight markers electrophoresed on the same gel are indicated on the left. The arrowheads on the right indicate the 0.9 kb, 1.6 kb and 2.5 kb species discussed in the text. The contents of each lane were as follows.

lane 1: non-transgenic DNA

lane 2: Founder D

lane 3: D1.1

lane 4: D1.4

lane 5: D1.6

lane 6: D1.7

lane 7: D.1.10

lane 8: D1.12

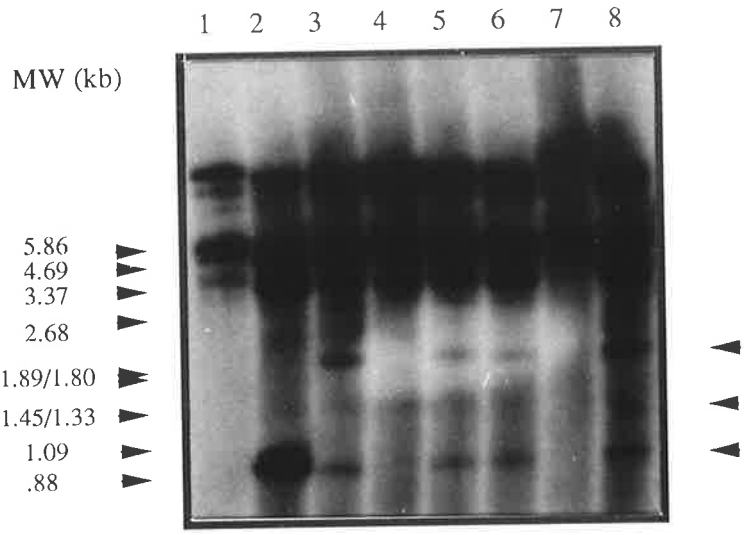
FIGURE 2.2.5 iii) Southern analysis on Eco RI digested genomic DNA (20 µgs) was performed as above on samples from the F1 generation of animal M5.5. This filter was probed with the Hind III-Ava I MTIIa promoter probe and washed at 42 °C in 0.1 x SSC/0.1 %SDS for 30 minutes before being autoradiographed for 72 hours. The sizes of SPP1 markers electrophoresed on the gel are indicated on the left and the 0.9 kb Eco RI fragment diagnostic of head-to-ail transgene integration is shown with an arrow on the left of the autoradiograph.

lane 1: non-transgenic rat

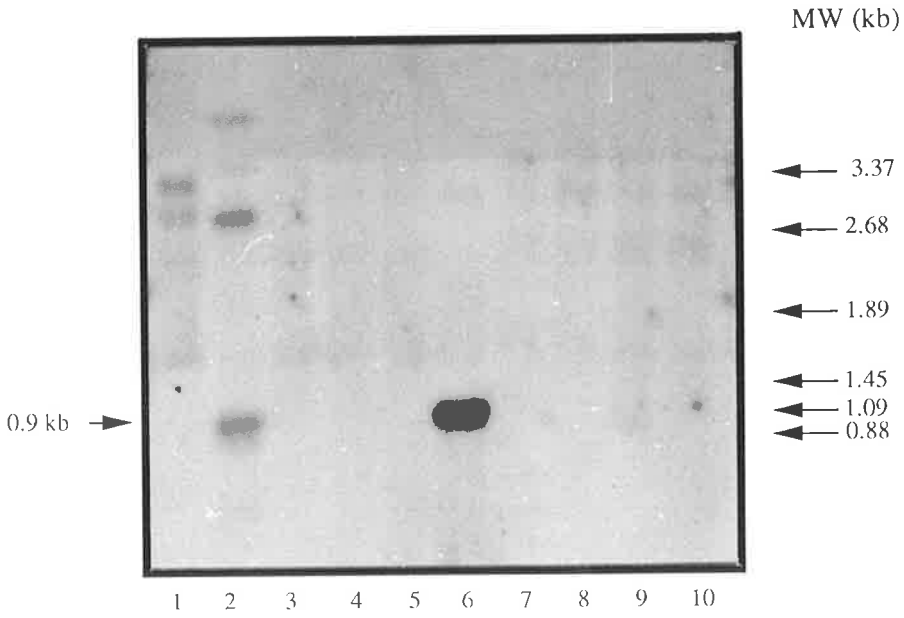
lane 2: Founder M5.5

lanes 3-10: Animals M5.5.1-M5.5.

EcoRI Southern Analysis Of D F1 Generation



Eco RI Southern Analysis of M5.5 F1Generation Animals



Characteristics of F1 and F2 Generation MTIIa-pGH rats

ID.NO.	SEX	plasma pGH (ng/ml)	Relative size Weight ratio
C1.13	F	717	1.72
D1.1	M	19	0.89
D1.5	M	14	1.03
D1.6	M	15	0.88
D1.7	M	16	0.86
D1.10	F	13	0.98
D1.12	F	22	0.95
M5.5.4	F	25	1.64
M5.5.4.1	M	49	1.65
M5.5.4.2	M	46	1.78
M5.5.4.3	M	37	1.74
M5.5.4.6	F	68	1.5

TABLE 2.2.5

Shows the plasma pGH levels and relative size of the F1 and F2 generation MTIIa-pGH transgenic rats. The weight ratio was calculated as the weight of the animal in question at 12 weeks of age divided by the mean weight of its sex-matched littermates at the same age. In the case of M5.5.4.1-M5.5.4.3 there were no non-transgenic male littermates from which to calculate the ratio. Hence in calculating the ratios for these animals the mean weight of the non-transgenic F1 males at 12 weeks of age was used.

FIGURE 2.2.5 iv) Eco RI Southern analysis was carried out on the F2 generation animals from the mating of D1.6. The filter was hybridised to the Hind III-Ava I fragment of the MTIIa promoter and was washed up to 42°C for 30 minutes in 0.1 x SSC/0.1 % SDS before autoradiography for 72 hours. The sizes of SPP1 markers are shown on the right hand side while the arrows at left highlight the 0.9 kb and 1.6 kb Eco RI fragments present in several animals.

lane 1: D1.6

lanes: 2-14: D1.6.1-D1.6.13

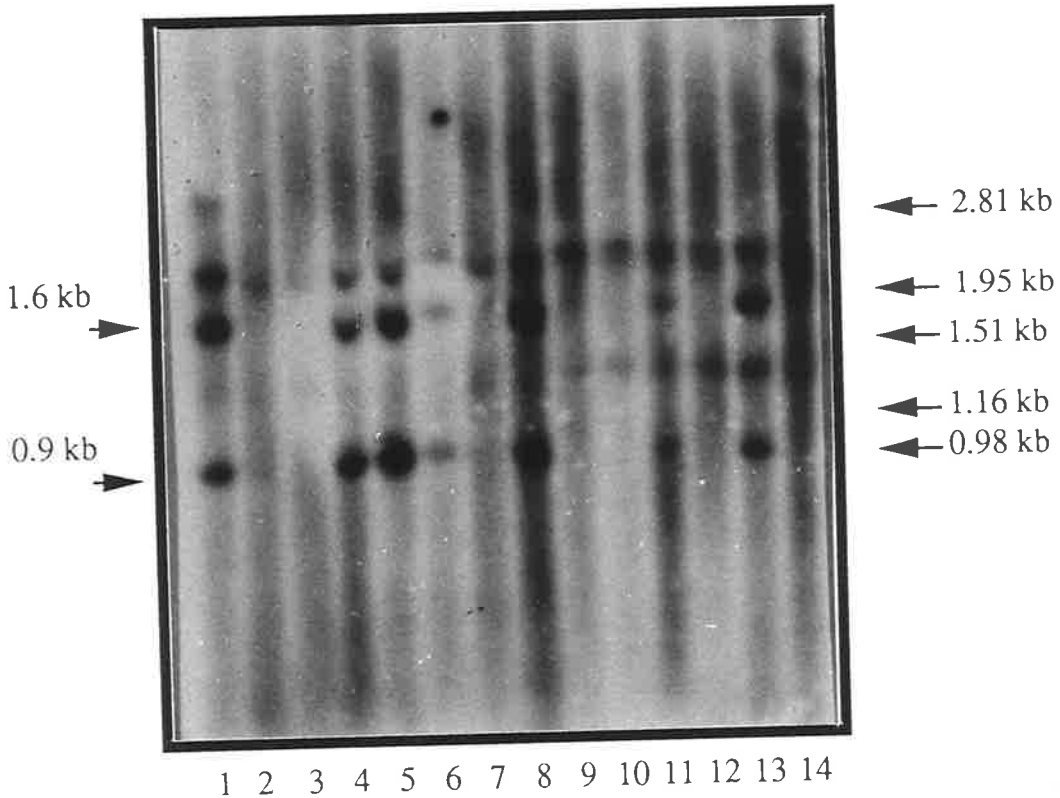

1.6 kb
0.9 kb
2.81 kb
1.95 kb
1.51 kb
1.16 kb
0.98 kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 2.2.5 v) Polymerase chain reaction (PCR) (see materials and methods section 6.3.13) was carried out on the F2 generation of M5.5.4 to quickly identify those offspring carrying the transgene. The two primers used were designed to amplify up a 130 bp portion of the human MTIIa promoter (primers 1367 and 1384). PCR reactions were carried out in a total volume of 50 µls. The following conditions were used for PCR.

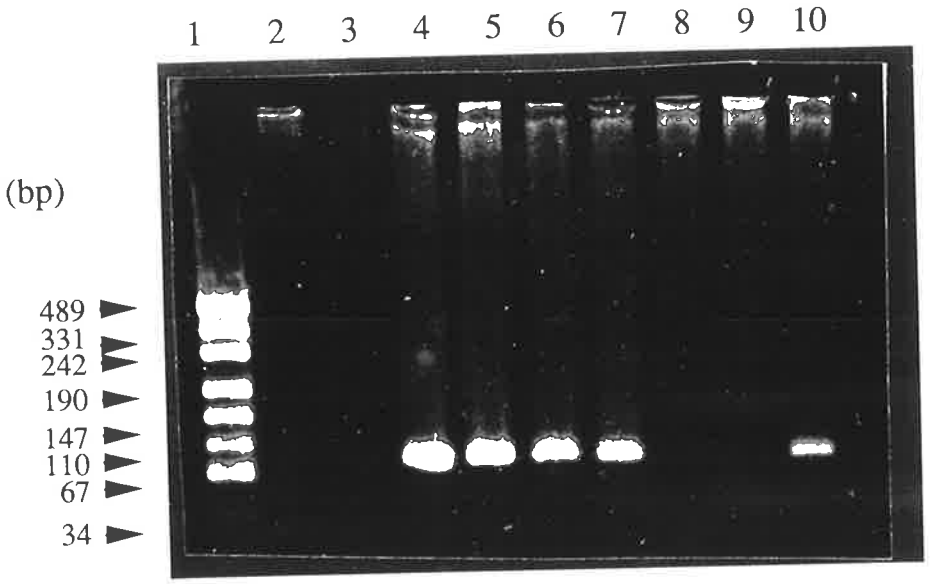
1 cycle: 2 minutes at 94°C (denaturing)

29 cycles: 30 seconds at 94°C/1 minute at 59°C (annealing)/1 minute at 72°C(extension)

5uls of each reaction was electrophoresed along with Hpa II cut pUC, DNA markers (lane 1) (low molecular weight range) on a 2 % Agarose TBE gel and the PCR product visualised by staining the gel in Ethidium Bromide.

In each PCR a tube was included lacking template DNA as a contamination control (track 2). In addition a negative control, containing non-transgenic rat DNA was included (track 3) and a positive control in this case DNA from M5.5.4 (track 4). Tracks 5-10 contain DNA from M5.5.4.1-M5.5.4.6.

PCR Identification of F2 generation MTIIa-pGH Transgenic Rats



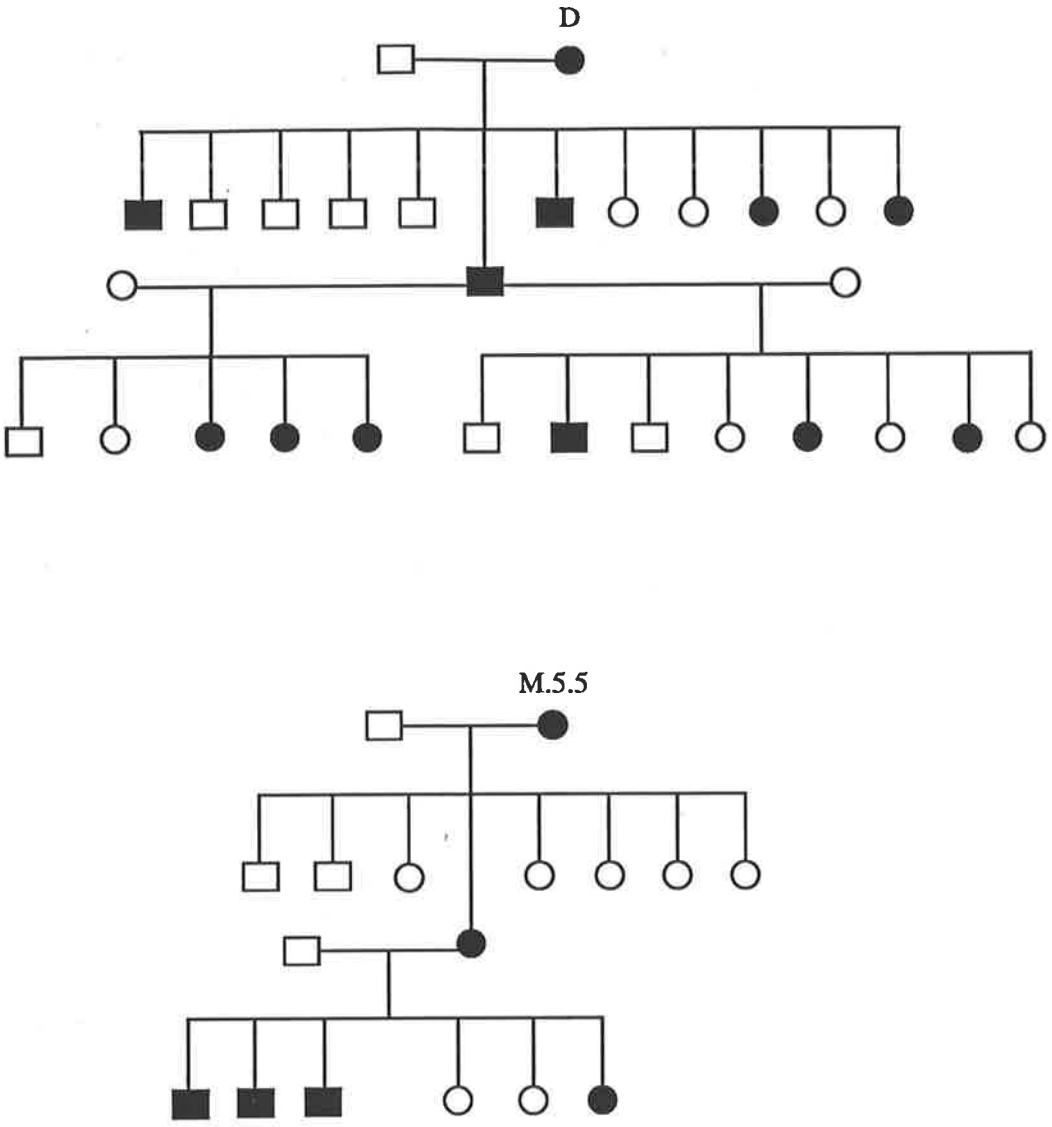


FIGURE 2.2.5 vi)

Shows the family trees of two MTIIa-pGH transgenic rats D (top) and M5.5 (bottom). Males are indicated by squares and females by circles. Shaded figures indicate animals which have the transgene as judged by dot-blot, Southern or PCR analysis. Shaded individuals have also been shown to express pGH by RIA.

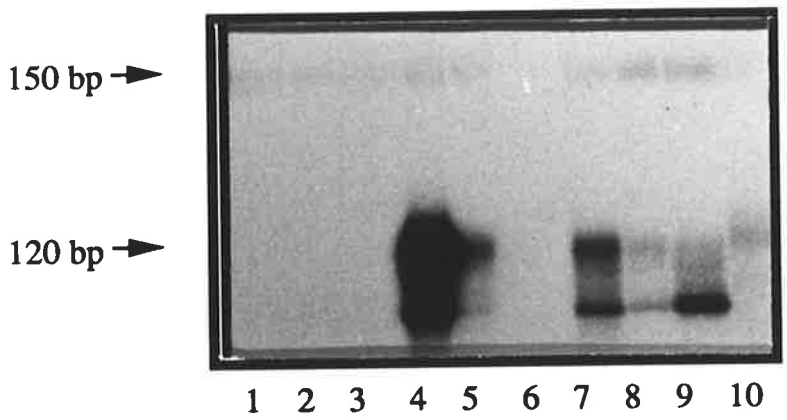
FIGURE 2.2.6 i) Ribonuclease Protection was carried out on RNA isolated from tissues of MT-1. 20 µg of each RNA was hybridised overnight with a probe complementary to the 3' untranslated region of the pGH gene. The full length probe is approximately 150 bps and protects a species of 120 bps in pig pituitary RNA (figure 2.2.6 ii) lane 15). Hybridisation was carried out at 50°C and hybrids were digested with both RNase A and T1 at 37°C for 30 minutes. The protected species were then separated on a 6 % polyacrylamide gel before autoradiography for 72 hours. The faint 150 bp band present in some lanes is due to incomplete digestion of the full length probe

lane 1: probe (with digestion)
lane 2-3: Liver RNA from non-transgenic rats
lane 4: MT-1 Liver RNA
lane 5: MT-1 Kidney RNA
lane 6: no sample loaded
lane 7: MT-1 Intestine RNA
lane 8: MT-1 Brain RNA
lane 9: MT-1 Muscle RNA
lane 10: MT-1 Heart RNA

FIGURE 2.2.6 ii) Ribonuclease protection analysis of transgene expression in MT-1, C1.13 and D. 50 µg of total RNA was analysed using the pGH 3' riboprobe. After digestion of the hybridised species with RNase A+T1, the protections were electrophoresed on 6 % polyacrylamide gels and visualised by autoradiography for 36 hours. The probe protects a species of 120 bps in pig pituitary poly A^+ RNA (lane 15). In addition a second smaller species is also protected. This smaller species can however be seen as a result of hybridisation between the probe and yeast RNA alone (lane 1) and may be be due to probe secondary structure. Furthermore this species is seen in RNA from non-transgenic rats and hence does not correspond to mRNA from the MTIIa-pGH transgene.

lane 1: probe + yeast RNA, **lane 2**: nothing loaded, **lane 3**: C1.13 Liver, **lane 4:** C1.13 Kidney , **lane 5**: C1.13 Intestine, **lane 6**: C1.13 Brain , **lane 7**: C1.13 Lung, **lane 8**: C1.13 Spleen, **lane 9**: MT-1 Liver, **lane 10**: MT-1 Kidney, **lane 11**: MT-1 Intestine, **lane 12:** D Liver , **lane 13**: D Kidney, **lane 14**: D Intestine, **lane 15:** pig pituitary poly A^+RNA, **lane 16**: non-transgenic rat liver

Expression of pGH mRNA in tissues
of rat MT-1



Expression of The MTIIa-pGH Transgene in
Different Transgenic Rats

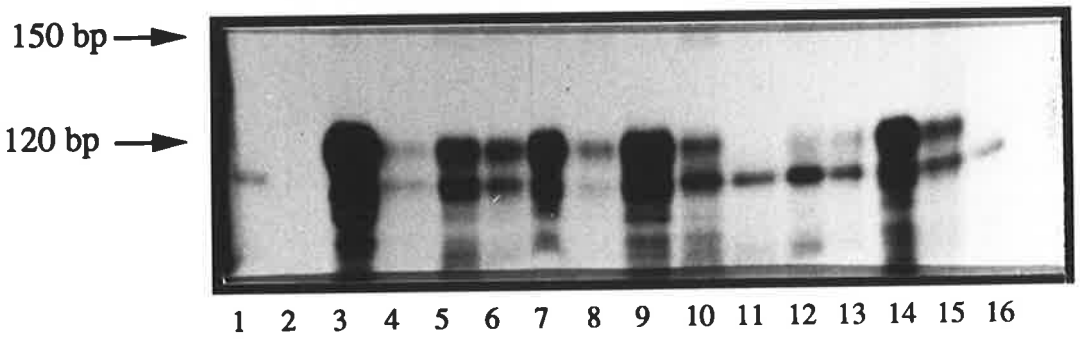


FIGURE 2.2.6 iii) Shows analysis of RNA from animals M1.9, M4.4 and M4.7. 50 µgs of total RNA was used in this Ribonuclease protection assay (conditions as per figure 2.2.6 i)). Autoradiography was carried out for 72 hours. Arrows indicate the 150 bp mature probe and 120 bp protected species respectively. Samples were as given below

lane 1: M4.7 Liver

lane 2: M4.7 Kidney

lane 3: M4.7 Intestine

lane 4: M4.7 Spleen

lane 5: M4.4 Liver

lane 6: M4.4 Kidney

lane 7: M4.4 Intestine

lane 8: M4.4 Spleen

lane 9: M1.9 Liver

lane 10: M1.9 Kidney

lane 11: M1.9 Intestine

lane 12: M1.9 Spleen

FIGURE 2.2.6 iv) Expression of the transgene in M2.1 was examined in the same assay as above using 50 µgs of total RNA. A separate gel was run to accomodate all samples in the assay Protected species in this case were visualised after overnight autoradiography due to the extremely high signal.

lane 1: probe alone (with digestion)

lane 2: pig pituitary poly$^+$ A RNA (0.5 µg)

lane 3: M2.1Spleen

lane 4: M2.1 Intestine

lane 5: M2.1 Kidney

lane 6: M2.1 Liver

lane 7: non-transgenic rat Liver

lane 8: non-transgenic rat Kidney

Expression of The MTIIa-pGH Transgene in Different Tissues of Transgenic Rats

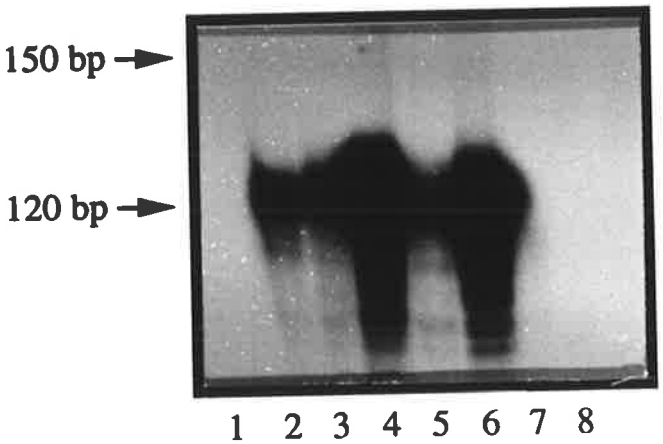


FIGURE 2.2.6 v) Ribonuclease protection was carried out on the F2 generation offspring from the D family of transgenic rats using the pGH 3' riboprobe employed in figure 2.2.6 i) (section 2.2.6). 50 µgs of RNA was used in this assay and autoradiography was for 72 hours. Arrows indicate the 150 bp mature probe and 120 bp protected species respectively.

lane 1: D Intestine

lane 2: D1.6.4 Liver

lane 3: D1.6.4 Intestine

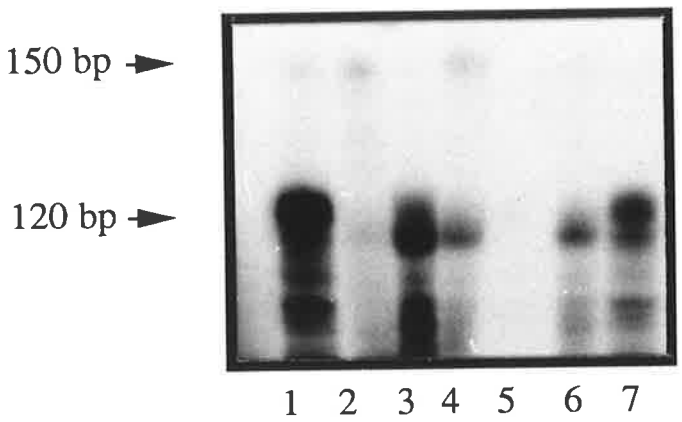
lane 4: D1.6.5 Liver

lane 5: D1.6.5 Intestine

lane 6: D1.6.12 Liver

lane 7: D1.6.12 Intestine

Expression of MTIIa-pGH in D Family



Zinc Induction of plasma pGH in D Family MTIIa-pGH rats

Rat No.	concentration of porcine GH in rat plasma (ng/ml)		
	Before Zn^{++}	After 14 days with Zn^{++}	7 days after Zn^{++} withdrawn
D1.1(m)	40	66	60
D1.2(m)	<17	25	<17
D1.3(m)	19	<17	<17
D1.4(m)	<17	20	<17
D1.5(m)	<17	<17	<17
D1.6(m)	42	83	78
D1.7(m)	31	43	39
D1.8(f)	20	<17	26
D1.10(f)	20	<17	<17

TABLE 2.2.7

Water containing 25 mM Zn++ was given to nine F1 generation animals of the D family for a two-week period in an attempt to induce expression of the MTIIa-pGH construct. Blood was taken from the animals prior to commencement of the diet, immediately following the diet and one week after withdrawal of the diet. Plasma samples were assayed for the presence of pGH (materials and methods section 6.3.22).(m) indicates male, (f) indicates female.

FIGURE 2.2.8 i) Plasma samples from C1.13, D, M1.9, M2.1 and two different non-transgenic control rats were diluted and heated in the presence of SDS before the equivalent of 1 µl of the original plasma was electrophoresed under non-reducing conditions, on a 12.5 % SDS polyacrylamide gel. The gel was subsequently electroblotted on to a nitrocellulose membrane and allowed to air dry before being probed with ^{125}I-IGF-II tracer overnight (materials and methods section 6.2.23). Following washing of the filter to eliminate background hybridisation, the filter was exposed to X ray film for 21 days. The size of ^{14}C molecular weight protein markers lane 1) are shown on the left of the autoradiograph. The samples loaded were as follows:

lane 2: non-transgenic

lane 3: non-transgenic (C1.12)

lane 4: C1.13

lane 5: D

lane 6: M1.9

lane 7: M2.1

Production of IGFBPs By MTIIa-pGH Transgenic Rats

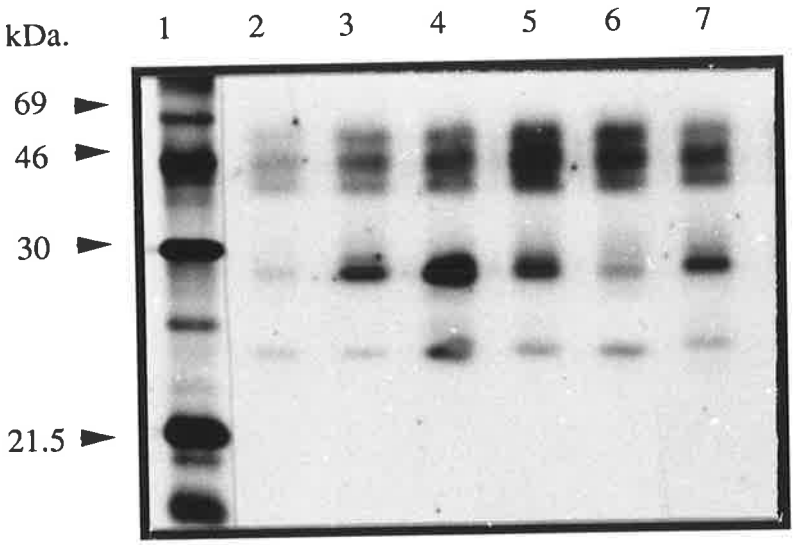


FIGURE 2.2.8 ii) 50 μg of total liver RNA was electrophoresed on 1 % agarose, phosphate buffered 10 % formaldehyde gels. Following capillary transfer to Nytran, the RNA was cross-linked to the filters using a Stratagene UV Crosslinker and the filters were probed with **a)** an Eco RI cDNA insert of rat IGF-1, **b)** rat BP-1 insert, **c)** riboprobe of the rat GH BP/receptor extracellular domain and **d)** 18s Ribosomal rRNA probe. After each probing, filters were stripped in 60 % formamide/ 2 x SSC at 65°C for 2-3 hours Complete stripping of the filter was monitored by autoradiography. The sizes of the RNA species are given with arrows on the left of the autoradiograph and were deduced from the positions of the bands relative to the 28S and 18S rRNA bands seen on the stained filter after transfer, in conjunction with radioactive DNA markers co-electrophoresed on the gel (not shown). The samples loaded on the gel were as follows:

lane 1: M2.1

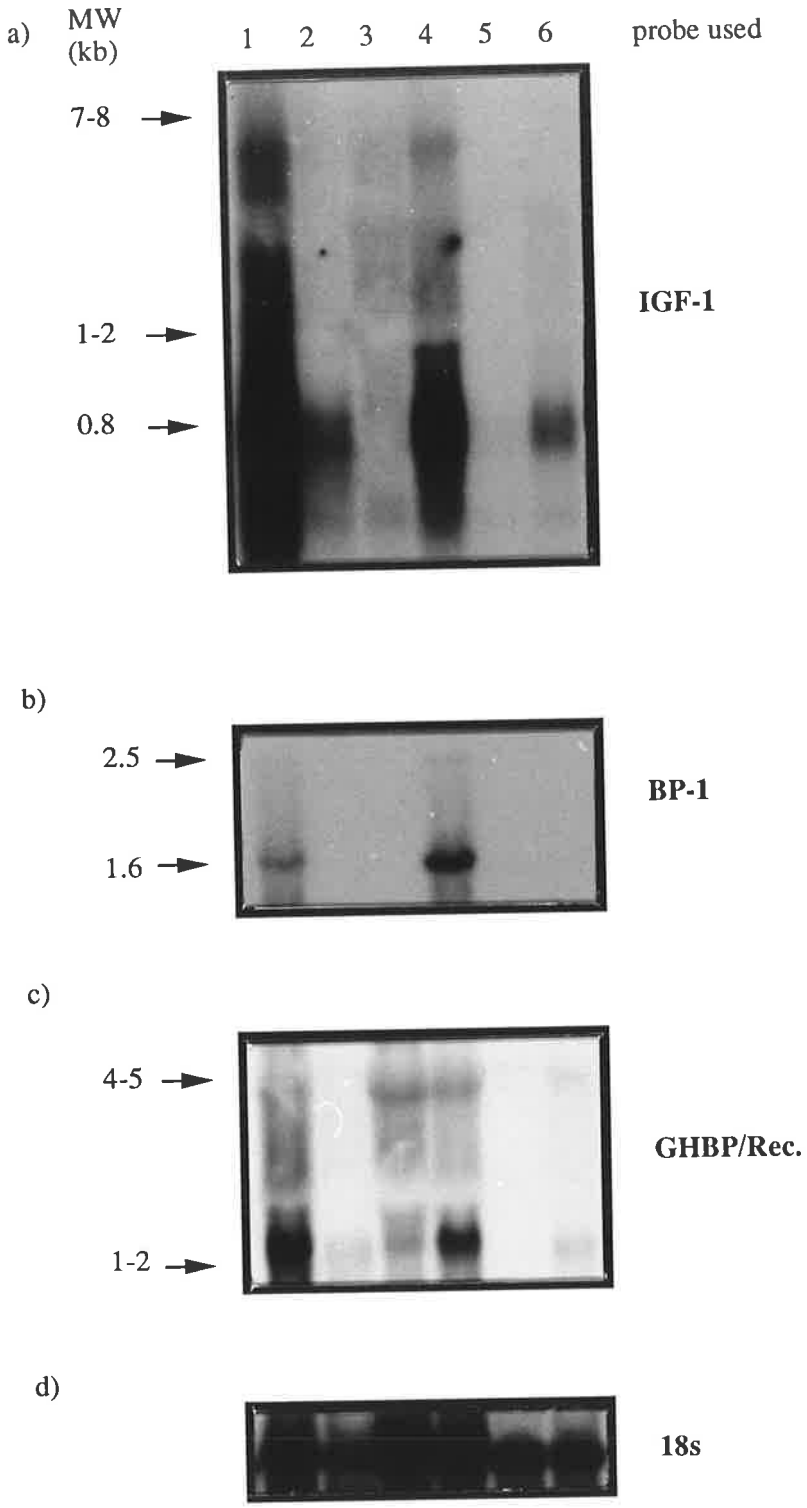
lane 2: M1.9

lane 3: D

lane 4: C1.13

lane 5: MT-1

lane 6: non-transgenic rat


a)
MW (kb)
1 2 3 4 5 6
probe used
7-8
1-2
0.8
IGF-1
b)
2.5
1.6
BP-1
c)
4-5
1-2
GHBP/Rec.
d)
18s

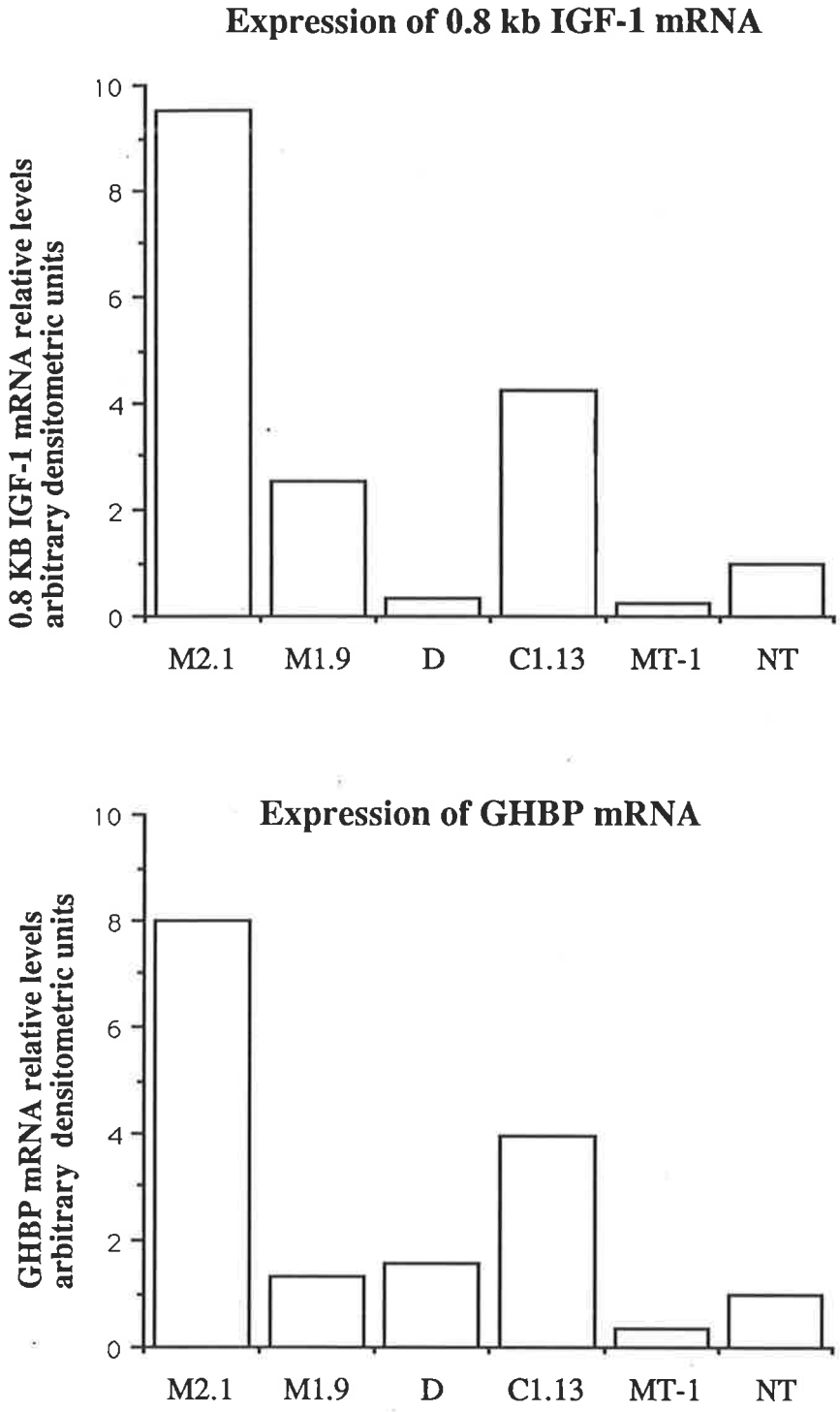


FIGURE 2.2.8 iii)

Densitometry of the autoradiographs shown in figure 2.2.8 ii) was carried out on a Molecular Dynamics Laser Densitometer using the Image Quant package. All readings were normalised against the loadings in each lane as deduced by the 18S probe. These graphs show the expression of the 0.8 kb IGF-1 mRNA and the GHBP mRNA in the individual transgenic rats relative to that seen for the non-transgenic control animal.

2.3 DISCUSSION:

With an embryo survival rate of 73% following microinjection, 56% integration of the MTIIa-pGH transgene into rats born from injected eggs after embryo transfer and 13% of the microinjected embryos developing into newborns, this study demonstrates that gene transfer into the germ-line of rats by microinjection can be accomplished as successfully as gene transfer in mice (Palmiter et al.,1986; Brinster et al.,1985). The integration rate achieved is greater than the 34 % seen with a HLA-B27 transgene in rats (Hammer et al., 1990) and compares favourably with the 62.5 % integration of the Ren-2 transgene into rats reported by Mullins et. al. (1990). Also this rate is considerably higher than the 30% integration of the MTIIA-pGH transgene into transgenic mice produced some years ago in this laboratory (Michalska A.E., Ph.D Thesis (1988)).

Animals produced in this study contained between 1-130 copies of the transgene per cell as judged by DNA dot-blot analysis (figures 2.2.2 i) and ii)). No direct correlation was found between the gene dosage and the level of expression achieved in any of the animals, consistent with the findings of others (Palmiter et al., (1983), Miller et al., (1989)). Of the expressing transgenic rats analysed, almost all appear to have integrated the MTIIa-pGH construct at a single site in the genome and with head-to-tail and head-to-head arrays of the construct at this site (see figures 2.2.3 i)-iii)). Head-to-tail arrangements of the transgene give rise to a characteristic 0.9 kb fragment upon Southern analysis with Eco RI, while head-to-head integrations produce a characteristic 1.6 kb Eco RI fragment. These results are similar to the results of transgenesis using this construct in mice (Vize P.D. Ph .D thesis, University of Adelaide 1987). Furthermore, the same pattern of integration has been maintained in F1 and in some cases F2 progeny, indicating the stability of the transgene in the germline (see figures 2.2.5 ii) and iii)). In the case of Founder A (see sections 2.2.3 and 2.2.5), it is possible that this animal had more than one integration site, with functional copies being present at only one locus in a head-to-tail array. The F1 generation appear not to have inherited the functional copies of the transgene at this locus from their expressing parent. Further Southern analysis would be necessary on DNA from this family determine the validity of this speculation. The low frequency of inheritance obtained in breeding some of the founder animals may be due to these animals being germ-line mosaics (e.g M5.5,C) as observed previously by Wilke et al., (1986.). In the case of pedigree M5.5, inheritance of expression does not seem to be sex-specific (see figure 2.2.5 vi). Thus incorporation of

the active transgene into an imprinted region (see introduction 1.6.2) of the rat genome cannot explain its expression pattern in this family. Palmiter et al. (1982) observed that achieving expression of their MT-TK fusion gene in mice from microinjection did not always ensure that the expression was stably inherited. Similar difficulty in achieving consistent inheritance of expression has also been observed for the MTIIA-pGH fusion gene in mice produced previously (Vize P.D., Ph.D thesis, University of Adelaide 1987)

In this study rats produced between 18-1200 ng/ml of pGH in their blood (see Tables 2.2.4 and 2.2.5), consistent with the levels observed in expressing transgenic pigs made with this construct (Vize et al , 1988). A correlation was found between the circulating levels of pGH in the females and their body size ($r=0.943$, $P<0.01$) (tables 2.2.4 and 2.2.5). However, the enhanced growth observed in our transgenic rats as a whole does not bear a direct relationship to the blood-borne level of the pGH protein. These observations have also been made for mice expressing hGH form the MT-1 promoter (Palmiter et al 1983). It should be pointed out that blood samples have not been taken routinely to determine the secretory pattern of the transgene derived pGH although expression from the MTIIa promoter would be expected to be constitutive. Hence it is possible that the pGH values used for correlation may be artifactually under- or over-estimated, preventing any significant correlation being made. Also, the tissue level of pGH expression may be more relevant to the growth of individual animals than the plasma pGH, since animals may express significant pGH in tissues other than the liver (the major source of plasma pGH)

There appears to be a threshold level of plasma pGH required to initiate noticeable enhancement of growth in these rats. The D family of animals express no more than 25 ng/ml of pGH in their plasma and all exhibit normal growth compared to controls, whereas other individual animals expressing upwards of 40 ng/ml appear to achieve enhanced growth relative to non-transgenic controls. It is feasible that this threshold is due to the sequestration of pGH by the rat GHBP present in excess in serum (Mannor et al (1991),Tiong et al (1991)). In this model, GHBP would sequester the low levels of pGH present in some of the animals preventing enhanced growth via the GH receptor. While this model may be applicable to the female transgenic rats there are two male rats, M1.2 and M5.2 (table 2.2.5) which produce significant pGH (85 ng/ml and 529 ng/ml respectively) and yet do not respond with increased growth. Although I have no explanation for this observation, Palmiter et al. (1983) have also observed transgenic mice with substantial immunoreactive hGH but lacking enhanced growth.

The enhanced growth I have observed in many of the transgenic rats (see table 2.2.4) has also been observed in studies in which intact rats were administered recombinant rGH at several doses. Groesbeck et al., (1987) administered rGH at either 0.4 mg/day, 1 mg/day or 5 mg/day to intact 4 week old female rats and observed body weight increases of up to 51% in rats treated with 1 mg/day and 73 % increases with the highest dose. These body weight increases ceased upon removal of the rGH Blood borne levels of rGH rose to approximately 350 ng/ml with the 1 mg/day dose and 1200 ng/ml after 20 days of treatment with the highest dose, 5 mg/day. In the MTIIa-pGH rats, plasma pGH levels of between 25 ng/ml-1200 ng/ml corresponded with body weight increases in female transgenic rats of 41%-100%, within the ranges observed for administration of rGH. Byatt et al (1991) have also shown that administration of bGH at doses ranging from 19 mg/day to 5 mg/day to mature 200 g female rats was able to stimulate significant increases in weight gain relative to vehicle treated controls, in line with our results using pGH. Interestingly these studies showed growth effects on female rats but no reports have been published on the effects of GH infusion on male rats. Due to insufficient numbers of breeding animals at this stage, it has not yet been possible to analyse the growth of individual tissues in any of the MTIIa-pGH expressors. Such measurements will enable more conclusions to be made concerning the mode of growth in these animals in the future.

As illustrated in figures 2.2.4 i)-iii), the enhanced growth of expressing transgenic rats appears to manifest itself prior to weaning, consistent with the expected expression of the MTIIa promoter at these earlier time points in development (Andrews et al 1991). This result is particularly interesting in light of the recent finding that mRNA and protein for the rat GH receptor and BP are present in the early embryonic tissues of the rat (Garcia-Aragon et al., 1992). In addition, a study by Palmiter et al (1983), suggested that hGH was produced from a MT-hGH transgene in mice from fetal stages, and that accelerated growth in these mice commenced from 16 to 22 days after birth. Studies are currently underway in our laboratory to examine more closely the onset of pGH expression and GH responsiveness in one family of transgenics.

In all fast growing transgenics examined by RNAse protection, the major tissue site of pGH expression was found to be the liver (figures 2.2.6 i)-iii)). It is not suprising therefore, that these faster growing animals also possessed higher plasma levels of pGH. In addition, pGH mRNA was observed in a wide variety of other tissues consistent with the housekeeping role of the MTIIa promoter (Durnam and Palmiter 1981; Karin and Richards

1982). The D line of transgenic rats exhibited lower plasma pGH levels than the faster growing lines (eg. M 5.5). Interestingly, the founder of this line (D) exhibited higher expression of the transgene mRNA in the intestine than the liver (figure2.2.6 ii)). Hence the lower plasma pGH levels recorded for this group of animals are probably due to their lack of liver-specific transgene expression. In support of this finding, animals in the F2 generation of this family (figure 2.2.6 v)) exhibit a similar expression pattern to D. If true for additional members of this family, intestinal as compared to liver-specific transgene expression in Founder D probably indicates that the D line of animals are unique due to insertion of the transgene at a specific chromosomal location which is able to confer different regulation on the integrated sequences. Effects of the position of chromosomal integration on the tissue site of expression of transgenes have previously been reported (Lacy et al., 1983). To assess the location of transgene integration, it would be necessary to perform chromosomal *in situ* hybridisation on metaphase chromosomes from animals in the D and other families.

The plasma levels of pGH in several animals from the D family have also been shown to be inducible by zinc (see table 2.2.7). At this time it is not known whether the growth of these animals is also zinc inducible, but experiments are currently being planned to address this point.. Similar anomalies between lines of expressing transgenic animals have been observed by Shamay et al., (1992) who saw differential hormonal regulation of Whey Acidic Protein transgenes in three lines of transgenic mice. Since animals from at least one other family (M 5.5) do not show expression changes in response to zinc (data not shown) it seems likely that the induction phenomena may be a characteristic specific for the D family.

In view of the infertility seen in female mice overexpressing hGH, rGH and bGH from the MT-I promoter (Bartke et al., 1988, Hammer et al., 1984, Naar et al., 1991), it is of interest that the majority of successful breedings carried out during this study used expressing transgenic dams. This suggests that in rats pGH is unable to substantially or detrimentally perturb the reproductive capacity of females. As pituitary secretion of GH is able to influence the production of reproductive hormones it would be interesting to examine the pituitary expression of endogenous rGH in these transgenic rats.

Plasma IGF-I levels were measured in four individual animals throughout the study (table 2.2.4). Three of these rats exhibited high plasma pGH levels and the fast growth phenotype (M1.9,M2.1 and M4.4) while D exhibited low plasma pGH and normal growth. As expected the two highest pGH expressors (M1.9 and M2.1) expressed largely from the

liver and exhibited high circulating IGF-I at the time of death. Coincident with high plasma IGF-I concentrations in these animals was an elevated level of liver IGF-I mRNA (figure 2.2.8 ii) a)) consistent with the Somatomedin Hypothesis. Furthermore figure 2.2.8 i) shows an increase in the levels of the 40-50 kDa IGFBP-3 associated species in both M1.9 and M2.1 presumably required to carry the increased level of plasma IGF-I. Also both ligand blotting (figure 2.2.8 i)) and Northern analysis (figure 2.2.8 ii) b)) show, increases in IGFBP-1 have occurred in M2.1. In contrast to this situation M4.4 shows little increase in liver IGF-I mRNA or plasma IGF-1 (574 ng/ml compared to 550-600 ng/ml for non-transgenic rats assayed) despite liver expression of pGH (figure 2.2.6 iii)) and relatively high plasma pGH, suggesting that expression of IGF-I may be have become refractory to GH in this animal or may be under the influence of other controls (e.g. nutrition). Similar states have previously been observed in diabetic rats (Scheiwiller et al., 1986), but are normally associated with inhibited growth. Animal D showed little increase in liver IGF-I mRNA consistent with the low liver expression of pGH in this animal. Interestingly, plasma levels of IGF-I, IGFBP-3 and IGFBP-1 (figure 2.2.8 i)) have been increased in this animal too. Increases in BP-1 are supported by Northern analysis (figure 2.2.8 ii) b)). High circulating IGF-I in this animal may be accounted for by GH stimulated expression of IGF-I in extrahepatic tissues (in particular the intestine), although this has not yet been shown by mRNA analysis.

In this study two phenotypically different groups of animals have been produced by microinjection of the same construct . One group appear to express primarily in the liver, exhibit variable levels of plasma pGH but respond to the hormone with enhanced growth throughout their life. The second group are essentially indistinguishable from control animals with respect to their growth and exhibit low levels of plasma pGH which are however, inducible in the presence of Zn^{++} in their drinking water. Expression of pGH in these animals appears to show a different tissue distribution. It is likely that these two groups of animals reflect differences in the chromosomal location of the introduced transgene, although this suggestion will require further experimentation to substantiate. Further detailed analysis of these two distinct groups of pGH expressing rats is ongoing and should permit us to examine in greater depth the mechanisms responsible for growth in the rat. Importantly, this study showed the amenability of rats to gene transfer and suggested that introduction of other constructs (in particular the PEPCK-IGF-I transgene) into this species would be feasible.

CHAPTER 3
PRODUCTION OF CHIMERIC PEPCK IGF GENES AND THEIR EXPRESSION *IN VITRO*

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3.1 INTRODUCTION:

The gene encoding human insulin-like growth factor-I spans at least 70 kb of DNA (Rotwein et al., 1986). Due to the enormous size of this gene it is not yet feasible to introduce it into the germ-line of animals using conventional microinjection procedures. However, two distinct types of cDNAs have been reported which encode the mature human insulin-like growth factor-I polypeptide (Jansen et al., 1983: Rotwein et al., 1986). These cDNAs termed type 1**a** and 1**b** differ at their 3' ends in the C-terminal sequence encoding the E peptide of the hIGF-I protein. Because of their small size (450-500 bps) relative to the genomic copy of the gene such cDNAs are viable alternatives for transgenesis studies. Indeed, transgenic mice have been produced by Mathews et al. (1988) using the hIGF-I **a** cDNA under the control of the metallothionein-I (MT-I) promoter. Expression of this cDNA has also been reported *in vitro* (Bovenberg et al., 1990). In our department, expression of a hIGF-I type **a** cDNA has been achieved using the strong Rous Sarcoma Virus LTR promoter (McKinnon et al., 1991). This study also reported the RSV driven expression and purification of des(1-3)IGF-I, an N-terminally truncated, superpotent derivative of IGF-I in Chinese Hamster Ovary cells (CHO cells).

With the eventual goal of achieving inducible expression of hIGF-I and/or des(1-3)IGF-I in transgenic rats during dietary or hormonal stress, it was necessary to produce a construct containing the IGF-I/des(1-3)IGF-I cDNA under the control of a promoter that would be appropriately regulated by diet and/or hormones. In addition, it was desirable to restrict expression to a specific set of tissues. Furthermore, as IGF-I is known to play an important role in animal development, it was necessary to choose a promoter which would be largely inactive before birth. For these reasons, the promoter of the rat cytosolic phospoenolpyruvate carboxykinase gene (PEPCK pr.) was chosen. This promoter has been studied extensively and has been shown capable of directing controllable expression of heterologous genes both *in vitro* and *in vivo* (see Introduction section 1.7).

Thus, this chapter describes the production of constructs containing the IGF-I and des(1-3)IGF-I genes linked to the rat PEPCK promoter. Also, the subsequent transfection of these constructs into tissue culture cells and their expression therein is examined.

3.2 RESULTS:

3.2.1 Construction of the PEPCK-IGF-Ia) transgene

The starting point for the construction of the PEPCK-IGF-Ia) transgene was two individual plasmids, pRSVIGF-I and BH 1.2 (shown in figure 3.2.1) which housed the IGF-I cDNA and PEPCK promoter sequences respectively.

Plasmid pRSVIGF-I has been previously employed to produce hIGF-I in permanently transfected Chinese Hamster Ovary (CHO) cells (McKinnon et al., 1991). This plasmid was a modification of the pAVOO9A+ expression vector (Choo et al., 1986) and contained strong viral promoter elements (Rous Sarcoma Virus , Long Terminal Repeat (RSV LTR)) as well as the neomycin gene to enable selection of antibiotic resistant (G418) cells that have incorporated the plasmid. The dihydrofolate reductase gene originally present in the vector pAVOO9A+ had been removed by Hind III digestion and subsequent re-ligation. The original pAVOO9A+ vector also contained a sheep metallothionein 3' region which had an atypical polyadenylation signal, AGATAAA. This fragment had been replaced by a 624 bp insert containing the 3' end of the hGH gene (downstream of hGH exon 4) as an end-filled Sma I/Eco RI fragment. The hGH 3' end contains a polyadenylation and associated processing signals which have been previously used to facilitate expression of cloned genes *in vitro* and *in vivo* (Luciw et al., 1983; Overbeek et al., 1986; Pfarr et al., 1986). In creating this insertion, the Eco RI site was destroyed (indicated in figure 3.2.1 i) by an Eco RI*) but the Sma I site was reconstituted. In addition, the vector pRSVIGF-I contains a human IGF-I cDNA. To obtain this clone a human liver cDNA library in lamda gt 11 (courtesy of Dr. G. Howlett, Department of Biochemistry, Melbourne University, Melbourne, Victoria, Australia) was screened by Dr. P. Krieg (University of Texas, Austin Texas, Dept. of Zoology) with a 30 mer oligonucleotide designed to be complimentary to bases 179-208 of the hIGF-I sequence reported by Jansen et al., (1983). Several identical clones were isolated which all contained an approximately 600 bp insert with the entire coding region of hIGF-I up to position -12 of the leader sequence. The extra bases coding for amino acids at -13 to -25 of the leader were added by site directed mutagenesis of the clones in a M13mp18 vector. Also, a Bam HI site four bases upstream of the intiating methionine was introduced allowing removal of the IGF-I cDNA as a Bam HI fragment.

The complete IGF-I sequence was then excised as a Bam HI/Bam HI fragment and ligated into the expression vector, behind the RSV promoter.

Plasmid BH 1.2 (figure 3.2.1 ii)) was kindly provided by Professor R.W.Hanson (Case Western Reserve University, Cleveland, Ohio) and contains a 1.2 kb genomic DNA fragment of the rat cytosolic PEPCK gene. This region contains 560 bp of 5' flanking DNA which extends into the second intron of the PEPCK gene and contains numerous regulatory sequences (Yoo-Warren et al ., 1983). Digestion of the plasmid with Bam HI and Bgl II yields a 620 bp fragment which has been used to direct tissue-specific, hormone and dietary-inducible expression of the bGH stuctural gene in transgenic mice (McGrane et al ., 1988). The Bgl II site is just inside the first exon of the PEPCK gene at position +73. This 73 bp of sequence is transcribed but not translated (McGrane et al., 1988). The PEPCK promoter region contained within plasmid BH1.2, in conjunction with reporter genes such as the herpes simplex virus thymidine kinase structural gene have been used to analyse the expression of this promoter in transfected hepatoma cells (Wynshaw-Boris et al., 1984). Hence this plasmid provided me with a source of rat PEPCK promoter sequences.

The cloning steps carried out in producing the PEPCK-IGF-I a) construct are shown in figure 3.2.2. Initially, the IGF-I cDNA was excised from pRSVIGF-I as a 448 bp Bam HI / Bam HI fragment. This insert was then cloned into the Bgl II site of BH 1.2. As the fragments generated by Bam HI and Bgl II have compatible ends, such a ligation is possible, but results in the destruction of both sites. In figure 3.2.2 the destruction of both sites is denoted by the B/B* site produced following ligation. BH 1.2 clones found to contain the 448 bp insert were sequenced using a 17 mer oligonucleotide primer (see section 6.2.8 and figure 3.2.8) in order to ensure the correct orientation of the IGF-I cDNA relative to the PEPCK promoter. This oligonucleotide binds the hIGF-I DNA from Glycine 32 of mature hIGF-I to Lysine 27 (figure 3.2.8) and is orientated 5'-3' towards the amino terminus of hIGF-I. Hence, using this primer, sequence could be read through the N-terminus of the IGF-I cDNA and into the adjoining BH 1.2 DNA Figure 3.2.3 shows the sequence around the insertion site of a BH 1.2 clone containing the IGF-I cDNA ligated directly behind the PEPCK promoter in the desired orientation for transcription of hIGF-I.

Clones with the cDNA in the correct orientation relative to the PEPCK promoter were digested with Bam HI / Hind III and the 1.6 kb inserts subcloned into pBluescript SK+ (pBS) to enable favourable restriction sites to be obtained for subsequent manipulation.

Bam HI / Hind III digestion releases an insert containing the PEPCK promoter linked directly to the IGF-I cDNA, with PEPCK gene sequences (exon/intron 1 and exon2/intron 2) at the 3' end of the cDNA. These extra PEPCK sequences were maintained for the purpose of transgenesis (see discusssion and chapter 4) and their inclusion denoted by the **a)** after the name of the construct. Subsequent constructs lacking these sequences were denoted with **b)** at the end of the construct name. Bluescript clones containing the 1.6 kb insert were digested with Bam HI / Cla I and the inserts subcloned further into pSP73 to enable restriction sites convenient for the final cloning step to be attained. Recombinant SP73 clones containing the insert were then digested with Sal I / Bgl II to remove the PEPCK-IGF-I fragment for directional cloning into the expression vector. In the final step, pRSVIGF-I was digested with Sal I / Bam HI to remove the RSVIGF-I gene from the hGH termination sequences and the Sal I./ Bgl II PEPCK-IGF-I fragment ligated in its place, creating pPEPCK-IGF-Ia). In doing so, both the Bam HI and Bgl II sites 3' of the insert were destroyed (again denoted in figure 3.2.2 as B/B*). Thus the final construct shown in figure 3.2.4 can be linearised with Sal I for transfection into cell lines or a transgene generated by double digestion with Sal I and Sca I.

3.2.2 Verification of the PEPCK-IGF-I a) construct

In order to verify that the construct was correct, several restriction digests were carried out on the final plasmid. Figure 3.2.5 shows the resolution of these digests on a 1 % agarose gel. Sal I, Bam HI, and Eco RI all digest pPEPCK-IGF-Ia) at a single site (lanes 2, 4 and 5) and therefore give rise to a single 7.3 kb species. Hind III digests the plasmid at two positions, 5' of the PEPCK promoter and 3' of PEPCK intron 2 (lane 3), giving rise to an approximately 2 kb fragment and a higher 5.3 kb species. Addition of Bam HI to the Hind III digest removes a small 360 bp fragment (only faintly visible near the bottom of lane 6) from the 2 kb insert generated by Hind III, leaving a 1.64 kb fragment in addition to the the 5.3. kb species (lane 6). Digestion of the plasmid with Eco RI / Sca I releases the 2.85 kb fragment and a 4.45 kb fragment (lane 7), while digestion of the plasmid with Sal I / Sca I releases a slightly larger transgene 2.96 kb insert along with a 4.34 kb higher species (lane 8). Digests in lane 9 and 10 are discussed below, in relation to figure 3.2.5 ii).

As a further verification of pPEPCK-IGF-Ia), several additional restriction digests were carried out and analysed by Southern analysis (see materials and methods section 6.3.15). Sal I, Sal I / Sca I, Hind III, Ava I and Dra I digests of pPEPCK-IGF-Ia) were

transferred to Nytran™ membrane and probed with the 540 bp PEPCK promoter Bam HI/Bgl II fragment (see figure 3.2.5 ii)). As expected the PEPCK probe detected a single 7.3 kb species following digestion with Sal I which cuts the plasmid only once (lane 1). Digestion of Sal I linearised plasmid with Sca I results in the production of a 2.96 kb PEPCK-IGF-Ia) transgene to which the PEPCK promoter probe hybridises strongly (lane 2). As mentioned above Hind III produces a 2 kb species, which contains the PEPCK promoter as evidenced by the strong hybridisation of the probe observed in lane 3 to this 2 kb species. Digestion of pPEPCK-IGF-Ia) with Ava I digests the plasmid in 3 positions and results in the production of two species of 3.8 kb and 3.2 kb respectively (see figure 3.2.5 i) lane 9) as well as a small 300 bp fragment not shown on the Southern. The PEPCK probe hybridises only to the smaller 3.2 kb species (lane 4) which contains the PEPCK promoter sequences. Dra I sites are only known with precision for the DNA included in the transgene. Within the transgene there are two Dra I sites, one is present within the PEPCK promoter (522 bps from the Bam HI site) while the second site resides in the hGH termination sequence (487 bps from the Sma I site). It is clear from digestion of pPEPCK-IGF-I a) (figure 3.2.5 i) lane 10) and pRSVIGF-I (data not shown) with this enzyme that many additional Dra I sites are present in the vector. Despite these sites, lane 4 shows that the predicted 1.63 kb fragment hybridises to the PEPCK probe as expected, confirming the nature of pPEPCK-IGF-Ia). Also a fragment of approximately 5.68 kb which represents the plasmid minus the 1.63 kb species is detected by the PEPCK probe. The hybridising species' between 1.63 kb and 5.68 kb therefore represent fragments generated by Dra I digestion 5' of the Dra I site within the PEPCK promoter. The presence of an intact 5.68 kb hybridising fragment therefore indicates that the Dra I digest was not complete.

3.2.3 Modification of the PEPCK-IGF-Ia) Transgene

At the time of commencement of this project a novel ten-fold more potent analogue of IGF-I had been discovered in bovine colostrum by our laboratory. Known as des(1-3) IGF-I, this analogue lacked the three amino terminal amino acids of IGF-I, viz. Glycine, Proline and Glutamate (Francis et al., 1986, 1988; Ballard et al., 1987). It showed increased biological potency *in vitro* by virtue of its lower affinity for the IGFBPs (Bagley et al., 1989; Szabo et al., 1988; Ross et al., 1989). This analogue has also been produced by permanent transfection of an RSV-des (1-3)IGF-I construct into Chinese Hamster Ovary (CHO) cells (McKinnon et al., 1991). Importantly, des (1-3) IGF-I has also shown

increased biological activity in some *in vivo* studies (Tomas et al., 1991). Hence, with the eventual aim of producing transgenic animals expressing this analogue inducibly, I have produced constructs which place des (1-3)IGF-I under the control of the PEPCK promoter.

i) Mutagenesis of the IGF-I cDNA

In order to produce these constructs, site directed mutagenesis (see materials and methods section 6.3.13) was carried out on pBS bluescript clones carrying the PEPCK-IGF-Ia) insert (see figure 3.2.2, step 2). The 30 mer used, RRS 644 (section 2.2.8) bound to the hIGF-I cDNA at Alanine 8 of mature IGF-I and proceeded in a 5'-3 ' direction up to Serine 21 of the 25 amino acid leader sequence (see figure 3.2.8). It lacked the sequences for Glutamate 3, Proline 2 and Glycine 1. Mutagenesis of these three amino acids was confirmed in the pBS clones by direct sequencing of the clones using a 17 mer oligonucleotide primer (shown in figure 3.2.8) which binds downstream of the mutagenic oligonucleotide. Figure 3.2.6 shows confirmation of the removal of the three, N-terminal amino acids in one particular bluescript clone of PEPCK-IGF-I compared with a non-mutated clone. Mutated inserts were then subcloned into SP73 and finally into the expression vector, as for the original PEPCK-IGF-I construct (see figure 3.2.2).

ii) Removal of PEPCK introns

Although, heterologous introns from the PEPCK gene had been included in the initial PEPCK-IGF-I construct to potentially enhance expression of our cDNA *in vivo,* it was also desirable to be able to compare expression achieved with these sequences to expression achieved in their absence. To this end the pBS PEPCK-IGF-I and PEPCK-des(1-3)IGF-I clones were subjected to additional mutagenesis to enable removal of the PEPCK sequences 3' of each respective gene. A 30 mer oligonucleotide (#1655 see materials and methods section 6.2.8) was designed to introduce a Hind III restriction site at the junction of the heterologous PEPCK sequences with the 3' end of the IGF-I or des(1-3)IGF-I genes (shown schematically in figure 3.2.7). By introducing a Hind III site at this position, the PEPCK sequences could be conveniently removed as a 560 bp Hind III fragment. Mutagenesis was carried out (see section 6.3.13) and mutagenised clones were selected on the basis of Hind III digests. Clones which gave a 560 bp fragment upon digestion with Hind III were kept. Following separation of the Hind III fragment from the remaining vector by preparative gel electrophoresis (see sections 6.3.4 and 6.3.6), the vector was

isolated and its Hind III ends religated (see section 6.3.7). Subsequently, the approximately 1 kb Bam HI / Cla I inserts containing either PEPCK-IGF-Ib) or PEPCK-des(1-3)IGF-1b) were isolated and subcloned into SP73. The remaining cloning steps were as described initially (see figure 3.2.2). Restriction digests have been carried out on all final constructs produced and have confirmed that all are as stated (data not shown). Furthermore, sequencing of clones subsequent to mutagenesis has confimed that the IGF-I and des(1-3)IGF-1 sequences were unaltered. Figure 3.2.7 shows schematically the four constructs produced in this study. Those constructs with the 3' PEPCK sequences removed are denoted with **b)** following their name.

3.2.4 Production of a hIGF-I Riboprobe to detect PEPCK-IGF-I mRNA

The insulin-like growth factor-I protein is highly conserved across evolution as is its corresponding gene (Shimatsu et al., 1987). This high level of conservation along with the large number of cell types which produce IGF-I meant that analysis of expression in cells transfected with PEPCK-IGF-I could be difficult using Northern analysis. Thus, before commencing transfections of the PEPCK-IGF-I a) construct into cells it was necessary to produce a probe which could discriminate hIGF-I from any other form of endogenous IGF-I mRNA produced by the transfected cells. In addition, the levels of expression from the transgene may have been low and therefore difficult to detect by Northern analysis. Hence, it was decided to produce a riboprobe that would enable a more sensitive ribnuclease protection assay (RPA) to be used in analysing expression from the transfected cells and in subsequent transgenic animals produced.

Comparison of the hIGF-I cDNA (see figure 3.2.8) and both the rat and mouse IGF-I type **a** and **b** cDNA sequences using FAST A, (from the GCG package of programs) revealed a number of mismatches despite the overall homology of greater than 77 %. The number of mismatches was greatest in the sequence coding for the C-terminal 35 amino acid E peptide of the IGF-I molecule. the hIGF-I cDNA also contains approximately 40 bp of untranslated sequence at the 3 ' end of the E peptide which also differed from the rat and mouse sequences considerably (figure 3.2.8)

The hIGF-I cDNA was present in pRSVIGF-I (see figure 3.2.1) and could be removed as an Eco RI / Sma I fragment. This fragment was removed and ligated into the polylinker of pSP 73. Figure 3.2.9 shows the resultant clone pSP73-IGF-I. Conveniently the hIGF-I cDNA used in these studies contains a Rsa I site at position 346 (out of 448 bp

total sequence). Therefore, linearisation of pSP73-IGF-I with Rsa I enables antisense transcription of the hIGF-I cDNA from the T7 promoter present in the vector. Thus, a 159 bp antisense mRNA can be generated that specifically covers the 3' untranslated and E peptide region of the hIGF-I cDNA. This probe should protect 102 bp of the hIGF-I mRNA species.

Figure 3.2.9 shows that the newly synthesised 159 bp riboprobe (lane 2) does indeed protect the correctly sized 102 bp species in total RNA isolated from a hIGF-I expressing cell line (lane 3) whilst detecting no transcripts in non-transfected cells and mouse liver RNA samples (lane 5 and 4 respectively), as predicted. There is also a lower molecular weight species of approximately 80 bp present in both lane 2 and lane 3. The absence of this species in lanes 4 and 5 and its presence in lane 2 suggests it is likely to represent protection of hIGF-I mRNA (in lane 3) by a degradation product of the 159 bp riboprobe.

3.2.5 Permanent Transfection of Constructs

Given that expression of the IGF-I cDNA had previously been achieved in Chinese Hamster Ovary Cells (McKinnon et al., 1991) it was decided to initially transfect the PEPCK-IGF-Ia) construct into these cells. However CHO cells were not highly gluconeogenic and as such may have been a poor choice for PEPCK promoter mediated expression of IGF-I. Hence, transfections were also carried out into Reuber H4IIE cells a highly differentiated rat Hepatoma cell-line. These cells have been used extensively in studies of PEPCK expression (Granner et al., 1983; Wynshaw-Boris et al., 1984; Forest et al., 1990) and therefore contain all of the necessary trans-acting factors for expression from the PEPCK promoter.

Initially, transfections were carried out into CHO cells only, using the PEPCK-IGF-Ia) construct. To ensure the transfection procedure was working correctly the RSV-IGF-I construct (McKinnon et al., 1991) was transfected into these cells in parallel. Large amounts of each construct were prepared and linearized with Sca I, which cuts at a single site 5' of either promoter (see figures 3.2.1 and 3.2.4). The linearised constructs were then purified by gel filtration chromatography (see 6.3.22) and quantitated, ready for electroporation. Electroporation of CHO cells was carried out as described in section 2.3.19 using 15 μg of each construct. In addition, to electroporations of RSV-IGF-I and PEPCK-IGF-Ia), a mock (no DNA) electroporation was also carried out. Following electroporation, selection was applied using the antibiotic G418 (450 μg/ml), a neomycin analogue. Seven

days after commencing selection, all cells from the mock electroporation had been killed and individual colonies had begun to be formed on plates containing cells from the RSV-IGF-I and PEPCK-IGF-I transfections. Four G418 resistant CHO colonies from transfection of RSV-IGF-I were picked and fifteen were picked from PEPCK-IGF-Ia) transfection. Individual colonies were grown up to confluence in 75 cm^3 flasks and RNA from each harvested after overnight incubation in the presence of serum-free medium containing dexamethasone (50 nM) a known inducer of PEPCK expression (Granner and Beale 1985).

Figure 3.2.10 shows Ribonuclease Protection analysis on RNA extracted from many of the clones isolated. This assay employed the 159 bp hIGF-I riboprobe (see section 3.2.4). RNA isolated from a known hIGF-I expressing cell-line was used as a positive control in this assay (lane 2) and shows the correct 102 bp protected species. This assay along with other RPA assays (data not shown) indicated that 2/4 of the RSV-IGF-I clones were expressing hIGF-I but only 1/15 PEPCK-IGF-Ia) colonies (clone O (lane 12)) picked, expressed hIGF-I mRNA. Considering equal amounts of RNA were used for all samples, the level of expression achieved in the PEPCK-IGF-Ia) expressor was significantly lower than that of the RSV-IGF-I clone. This is perhaps not suprising given the strength of the RSV LTR promoter (Yamamoto et al., 1980).

Following the low rate of hIGF-I expression achieved in the initial transfections and with the production of PEPCK-des(1-3) constructs (see figure 3.2.7) now completed, a second round of transfections were conducted. This time, both CHO and rat Reuber H4IIE hepatoma cells were transfected with the four constructs shown in figure 3.2.7. Mock electroporations were again performed for each cell line but the RSV-IGF-I construct was not included for transfection because the rate of transfection as judged by the initial experiment was satisfactory. The conditions used for electroporation of H4IIE cells (see section 2.3.19) were based on the results of G. Bradiotti (Department of Biochemistry, University of Adelaide, Ph.D. Thesis 1991) and included the use of carrier DNA during the electroporation. Furthermore, selection of transfected H4IIE clones was carried out in a higher concentration of G418, 500 µg/ml, due to the high natural resistance of these cells. This level of antibiotic selection has also previously been reported by Forest et al., (1990), who produced permanent H4IIE cell lines containing PEPCK-CAT (Chloramphenicol Acetyl Transferase) constructs. One week after transfection all of the cells from the mock electroporations had died off, implying that this selection regime was successful. I picked

and grew up 28, G418-resistant CHO colonies, 7 for each of the four constructs transfected. Also 34, G418-resistant H4IIE clones were selected for expansion (7-9 for each construct).

As with the initial transfections each clone was grown to confluence in 75 cm^3 flasks and then overnight in the presence of dexamethasone (50 nM) to induce expression of the IGF-I gene. RNA harvested from each clone was subject to analysis by RNAse protection using the 159 bp hIGF-I riboprobe. Clones from transfection of the PEPCK-IGF-Ia) and PEPCK-IGF-I b) constructs are designated with the letters A, B / F, G for CHO cells and H4IIE cells respectively. Clones from transfection of PEPCK-des(1-3)-IGF-I a) and PEPCK-des(1-3)-IGF-I b) are given the letters C, D / H, I for CHO cells and H4IIE cells repectively.

Figure 3.2.11 i) and ii) show assays carried out on CHO and H4IIE clones respectively. In both assays the mature probe (159 bp) is still evident in each track due to incomplete digestion with RNAse. While the expected 102 bp species is absent from both normal CHO and H4IIE cells (figure i) lane E and figure ii) lane J) it is present in a number of clones from each construct (notably A3, B2, B3, C1, C2, C6, D3, D6, F2, F3, G8, H4, H5, I1, I3). However, the levels of hIGF-I were extremely low especially in H4IIE clones (the autoradiographs in ii) was exposed for 7 days) given the sensitivity of ribonuclease protection assay (Melton et al., 1984).

Although all clones screened by ribonuclease protection were G418-resistant and therefore carried the neomycin resistance gene from the transfected constructs, dot-blot analysis was carried out on several of the clones from each construct to ensure all contained DNA from the plasmids. The dot-blot shown in figure 3.2.12 i) was probed with a 487 bp Sma I/Dra I fragment from the 3' end of the hGH termination signal. This figure shows that the probe cross-reacts highly with sequences present in the CHO cell line. In spite of the background hybridisation observed in the non-transfected CHO cells (E),(row b lane 2) the signal observed in transfected clones (row c) is an order of magnitude higher indicating the presence of the hGH termination signal in these clones (in most cases between 24-32 copies of this sequence). In comparison the probe shows little cross-reactivity with sequences from non-transfected H4IIE cells (J) (row b lane 3). H4IIE transfectants (rows d and e) all appear to have integrated less copies of the plasmid (between 2-8 copies) than the CHO cells. Difficulty in transfecting this cell-line has previously been reported (Forest et al., 1990). All isolated clones have maintained their G418 resistance for several months in culture indicating that stable integration of the plasmid in each case has been achieved. Several clones were

grown for a period of 3 months in the absence of antibiotic selection. Upon reintroduction of selective media no alteration in growth or death of cells was observed, supporting the stability of plasmid integration. Furthermore, PCR analysis shown in figure 3.2.12 ii), using primers specific for the PEPCK promoter region and IGF-I/-3N leader region confirms that all clones have these sequences. These primers amplify a 285 bp region between the PEPCK promoter and the IGF-I leader peptide in DNA samples derived from clones A7, B2, C1, D1, D4, F3, G3, H4, II and I4 but do not amplify this fragment in DNA samples from non-transfected CHO or H4IIE cells (lanes 3 and 4) confirming intact insertion of this junction region.

Having shown the presence of the hIGF-I mRNA in a number of the CHO cell lines, all were re-grown up to confluence in 75 cm^3 flasks. Each flask was washed in two changes of PBS at 37 ^{o} C for one hour to remove any remaining serum from the flasks, before being incubated overnight in 10 ml of F12 medium containing dexamethasone (50 nM). Longer incubations in the presence of dexamethasone were not possible due to the large increase in cell death accompanying such treatments. Following 24 hour incubation, the medium was removed from the cells, briefly centrifuged to pellet any remaining cell debri and concentrated 8-fold by drying. These supernatants were then tested for their ability to stimulate protein synthesis as measured by incorporation of ^{3}H-Leucine into semi-confluent monolayers of rat L6 myoblast cells. The details of this assay are given in section 6.3.21.

Figure 3.2.13 shows the stimulation of protein synthesis in L6 myoblasts by supernatants from several CHO clones compared with the levels achieved in the presence of increasing concentrations of hIGF-I. These clones were the only ones to show significant stimulation above control levels in this assay. This result shows that protein synthesis was stimulated slightly (34 % above control) by the supernatants of non-transfected CHO cells (lane E) suggesting the secretion of endogenous growth factors by these cells during the overnight incubation period. Importantly, supernatants from transfected cells showed greatly enhanced stimulation (72-170 % above control) of protein synthesis implying the production of hIGF-I or des(1-3)-IGF-I by these individual clones. It should be noted that none of the supernatants from clones transfected with the PEPCK-IGF-Ib) construct were found to stimulate protein synthesis and are therefore are not included in figure 3.2.13. Interestingly, higher levels of protein synthesis were stimulated by supernatants derived from clones containing the PEPCK-des(1-3)-IGF-I a) and b) constructs, indicative of the higher biological activity of the des(1-3) peptide on L6 cells (McKinnon et al., 1991; Francis

et al.,1988). Thus, in addition to producing hIGF-I mRNA, biologically active material is being secreted, consistent with the production of hIGF-I or des(1-3)-IGF-I by these CHO clones. Similar analysis of supernatants derived from H4IIE cells showed little stimulation of L6 protein synthesis, consistent with the extremely low levels of hIGF-I transcription observed for these clones.

Attempts have been made to increase the expression of hIGF-I by both the CHO and H4IIE cell lines (data not shown) but all have been unsuccessful, including increasing the concentration of dexamethasone to 200 nM, harvesting RNA at earlier time points after incubation with inducer and inducing cells grown to different levels of confluence. Furthermore, expression of the neomycin resistance gene was examined by ribonuclease protection to ensure that the integrated sequences were still present. This assay (data not shown) confirmed that the neomycin resistance gene was expressed in the H4IIE clones indicating no loss of the integrated sequences had occurred.

3.2.6 Analysis of a PEPCK-IGF-I a) expressing CHO cell-line

As one permanent cell-line was expressing significant hIGF-I mRNA it was decided to analyse this expression in greater detail. To ensure that hIGF-I was being secreted into the medium by this cell-line, the expressing PEPCK-IGF-Ia) clone (1O), an expressing RSV-IGF-I clone a non-expressing PEPCK-IGF-Ia) clone (1K) and non-transfected CHO cells were each grown to confluence, before the serum containing medium was removed and each clone grown overnight in the presence of dexamethasone (50 nM), to induce PEPCK expression. Both the medium and the cell pellet were collected for analysis. RNA was extracted from the cell pellet using the method of Chomczynski and Sacchi, (1987) and ribonuclease protection assay carried out as shown in figure 3.2.10, confirming that only clone 1O and the RSV-IGF-I clone were producing hIGF-I mRNA (data not shown).

The medium was removed after overnight incubation and centrifuged briefly to remove cell debris. Subsequently, 300 µl of each medium was de-fatted by FREON extraction and subjected to gel filtration chromatography under acidic conditions (section 6.3.20). Acidic conditions were required to dissociate any IGFBPs from the secreted IGFs prior to radioimmunoassay (see section 2.3.22 and 4.2.5). Four pools of eluent were collected during the chromatography (pool 1: 6.5-8.5 ml, pool 2: 8.5-9.0 ml, pool 3: 9.0-11.0 ml, pool 4: 11.0-11.5 ml). Radioactive hIGF-I was chromatographed before and after the four samples and shown to elute as a single peak at 10.4 ml. Following chromatography

aliquots of each eluted pool were assayed in an IGF-I radioimmunoassay (see section 6.3.20) utilising a monoclonal antibody to hIGF-I provided by Dr. R. Baxter (University of Sydney) (Tomas et al., 1992).

Figure 3.2 14 shows the result of this assay. The immunoreactivity detected in pool 1 (6.5-8.5 ml) represents interference in the assay by IGFBPs present in these fractions which are able to sequester the ^{125}[I]-IGF-I tracer used in the assay and thus register as immunoreactivity. This figure shows clearly that pool 3 (9.0-11.0 ml) contains significant immunoreactive hIGF-I in samples derived from both the RSV-IGF-I clone and PEPCK-IGF-I a) clone 1O above the background level observed in the other two samples. Importantly, little immunoreactivity was observed in pool 2 indicating complete separation of IGFBPs from the IGFs. This assay showed that the RSV-IGF-I clone was expressing **264 ng/ml** hIGF-I while the PEPCK-IGF-Ia) clone (1O) expressed **191 ng/ml** hIGF-I consistent with the lower level of hIGF-I mRNA observed in the latter.

Aliquots of conditioned medium from this experiment were concentrated four-fold and assayed for their biological activity in an L6 protein synthesis assay (see section 6.3.21). Figure 3.2.15 shows that the hIGF-I secreted by PEPCK-IGF-I a) clone 1O is indeed biologically active. The stimulation of protein synthesis achieved with conditioned media from the PEPCK-IGF-I a) clone (206 % above control) was comparable to that observed with conditioned media from the RSV-IGF-I clone (228 % above control). Both were significantly greater than the 52 % stimulation above control observed in the presence of conditioned media from non-transfected CHO cells. Thus, the hIGF-I secreted by the PEPCK-IGF-I a) cell-line was biologically active.

In order to determine if the correct sized mRNA species was being produced by the PEPCK-IGF-Ia) expressing cell-line, RNA was harvested from non-transfected CHO cells, the RSV-IGF-I cell-line and the PEPCK-IGF-Ia) cell-line after overnight incubation in serum-free medium. Figure 3.2.16 i) shows Northern analysis of these RNA samples. The filter was probed with the Bam HI/Bam HI hIGF-I cDNA and was washed at high stringency. This probe detected an approximately 1.1 kb mRNA species in both the RSV-IGF-I and PEPCK-IGF-Ia) RNA but no species were detected in RNA from non-transfected cells. The RSV-IGF-I and PEPCK-IGF-Ia) constructs are predicted to produce mRNAs representing 1109 bp and 1435 bp (see figure legend 3.2.16) upon initiation of transcription from the RSV and PEPCK promoters respectively. The prediction for the PEPCK-IGF-Ia) mRNA species assumes that PEPCK exonic sequences present 3' of the IGF-I cDNA

would be included in the mature cytoplasmic transcript. The result observed from Northern analysis suggests that this region has been removed from the mature transcript giving rise to a species representing 1141 bp. Thus the 28 bp difference between RSV-IGF-I and PEPCK-IGF-Ia) transcripts would not be detected in this analysis. Re-probing of the filter with a rat 18 S rRNA probe confirmed that RNA was present in all lanes.

In order to examine the inducibility of hIGF-I expression in CHO 1O this clone was subject to treatment with dexamethasone and insulin, a potent inducer and potent inhibitor of PEPCK gene transcription respectively (Granner et al., 1983 ; 1985). In the ribonuclease protection assay shown in figure 3.2.16 ii) the 159 bp hIGF-I riboprobe (see figure 3.2.9 i)) was employed on RNA samples from PEPCK-IGF-Ia) clone 1O treated with F12 medium containing either 10 % serum, no serum, 100 nM dexamethasone, 200 nM dexamethasone or 100 nM dexamethasone plus 1 ng/ml insulin. This result shows that expression of hIGF-I mRNA was increased when serum was removed from the F12 medium in line with the presence of inhibitors of PEPCK expression (primarily insulin) in serum. Little effect on hIGF-I mRNA expression was observed in the presence of the lower concentration of dexamethasone whereas in the presence of the higher concentration of dexamethasone, hIGF-I mRNA levels were modestly increased. Furthermore, when insulin and dexamethasone (high dose) were added simultaneously levels of hIGF-I mRNA were decreased. These results although not quantitatively similar to the changes in PEPCK mRNA expression observed in H4IIE cells, are qualitatively similar and indicate that hIGF-I expression in this cell-line is controlled by the sequences of the rat PEPCK promoter.

3.2.7 Examination of H4IIE cells as a suitable model for expression of PEPCK-IGF transgenes

As mentioned previously, only low levels of expression were observed in H4IIE clones shown to contain PEPCK-IGF-Ia) or b) constructs. Since transcription of the PEPCK gene in this cell-line has been shown to be inhibited rapidly by insulin (Sasaki et al., 1984), it was possible that such inhibition could also be accomplished by IGF-I. Hence in order to test this hypothesis, the regulation of the endogenous H4IIE PEPCK gene was examined by ribonuclease protection.

In the experiment shown in figure 3.2.17 ii), H4IIE cells were grown to confluence to maximise PEPCK expression as shown by Beale et al., (1991). Serum containing medium was removed and cells were washed in PBS prior to overnight incubation in the presence of

DMEM containing either dexamethasone, insulin or increasing concentrations of recombinant hIGF-I. RNA was subsequently extracted and analysed by ribonuclease protection assay using a 189 bp antisense riboprobe covering the first 73 bps of the rat PEPCK gene. The construct for preparation of this riboprobe was produced by directional cloning of a 529 bp Eco RI / Bgl II fragment from pBH 1.2 (see figure 3.2.1) containing the PEPCK promoter, into the polylinker of pSP72 (see figure 3.2.17 i)). The construct was linearised with Nco I prior to transcription with T7 polymerase.

Figure 3.2.17 ii) shows that the probe protects two distinct species of approximately 70-75 bp in RNA harvested from H4IIE cells grown in the absence of serum (lane 2). Wynshaw-Boris et al., (1984) showed that the end-labelled Nco I-Bgl II PEPCK promoter fragment protected fragments between 70-75 bp, with the most intense being 73 bp using S1 nuclease mapping to identify the start site of PEPCK transcription. Thus, results presented here are in general agreement. Incubation of the H4IIE cells in the presence of dexamethasone (lane 3) has led to an approximately two-fold induction of PEPCK mRNA. As expected, treatment with insulin (lane 4) resulted in a large reduction (approximately five-fold) in PEPCK mRNA. Interestingly, treatment of the H4IIE cells with increasing concentrations of hIGF-I was also shown to reduce PEPCK mRNA. This presumed inhibition of PEPCK transcription was evident with as little as 10 ng/ml hIGF-I (lane 6). At the highest concentration of hIGF-I tested, 50 ng/ml (lane 7), the reduction in PEPCK mRNA was approximately four-fold. Thus, this experiment clearly showed that hIGF-I was able to inhibit the production of PEPCK mRNA in H4IIE cells. A further experiment on the H4IIE cells was performed to examine the expression of the PEPCK gene in the presence of IGF-I and dexamethasone applied concurrently. Again, RNA was harvested from the cells after overnight incubation in the presence of 50 nM dexamethasone (the same concentration used to induce my permanent transfectants) and increasing concentrations of hIGF-I or a single dose of insulin.

Figure 3.2.18 shows ribonuclease protection analysis carried out on 30 μg of RNA from each treatment using the 189 bp PEPCK promoter riboprobe. Only the protected 70-73 bp species are shown. Scanning densitometry of the protected species was carried out and the results are presented graphically below the autoradiograph. The level of PEPCK mRNA was increased 3-fold by treatment with dexamethasone (lane 2). This increased expression was maintained in the presence of hIGF-I at 5 ng/ml (lane 3). However, at a hIGF-I concentration of 10 ng/ml (lane 4), dexamethasone-stimulated PEPCK expression was

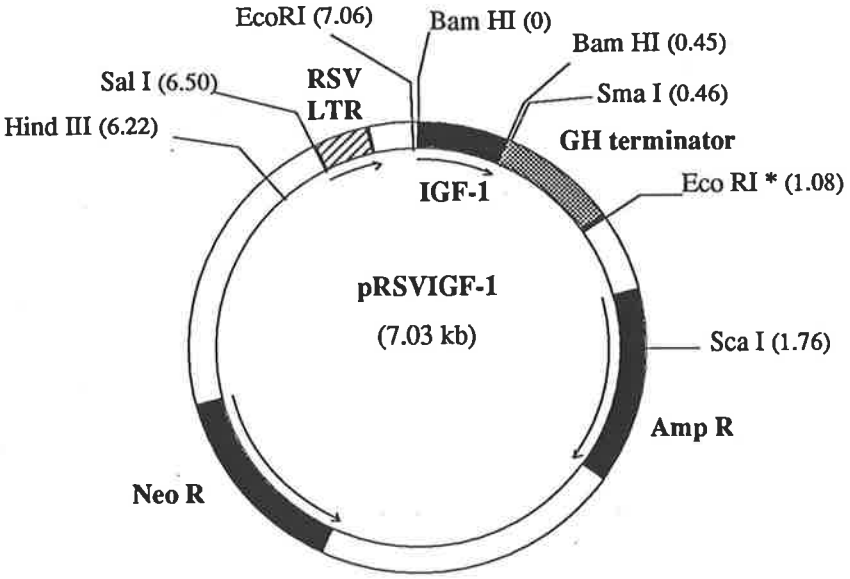
decreased by 24 % and with 50 ng/ml hIGF-I (lane 5) by 52 %. Treatment of the cells with insulin in the presence of dexamethasone virtually abolished production of PEPCK mRNA. Thus, although hIGF-I was not as potent as insulin in preventing expression of the PEPCK gene, as little as 50 ng/ml was sufficient to reduce PEPCK mRNA even in the presence of dexamethasone.

Therefore, these experiments suggest that the inability to produce H4IIE clones expressing the PEPCK-IGF-I a) or b) constructs is most likely due to the turning off of construct transcription by hIGF-I secreted into the surrounding media in the first 24 hours. Hence, despite possessing all the desirable characteristics for expression fom the PEPCK promoter these cells were inappropriate for studies on the regulation of the constructs I have produced.

FIGURE 3.2.1 **i)** Plasmid pRSVIGF-I contains a hIGF-I cDNA (448 bp) linked to the Rous Sarcoma Virus LTR promoter sequences (540 bp). Included in this construct is the hGH 3' transcriptional termination signal (624 bp). As well as containing the Ampicillin Resistance gene for manipulation in bacteria this plasmid contains the aminoglycoside phosphotransferase (Neo ^{R}) gene permitting selection of permanently transfected mammalian cell lines with the neomycin analogoue G418. This gene is expressed in eukaryotic cells by virtue of the SV40 early promoter. Eco RI* indicates that this site has been destroyed.

ii) Plasmid BH 1.2 is a pBR322 based vector and was kindly provided by Professor R.W. Hanson (Case Western Reserve University, Cleveland, Ohio). It contains 1.2 kb of the rat Phosphoenolpyruvate Carboxykinase (PEPCK) gene including 574 bp of 5' regulatory sequences which extends into the second exon of the PEPCK gene. The PEPCK initiation site containing73 bp of the first PEPCK exon is shown. This region is transcribed but not translated.

i)


EcoRI (7.06)
Bam HI (0)
Bam HI (0.45)
Sal I (6.50)
RSV
LTR
Sma I (0.46)
Hind III (6.22)
GH terminator
IGF-1
Eco RI * (1.08)
pRSVIGF-1
(7.03 kb)
Sca I (1.76)
Amp R
Neo R

ii)

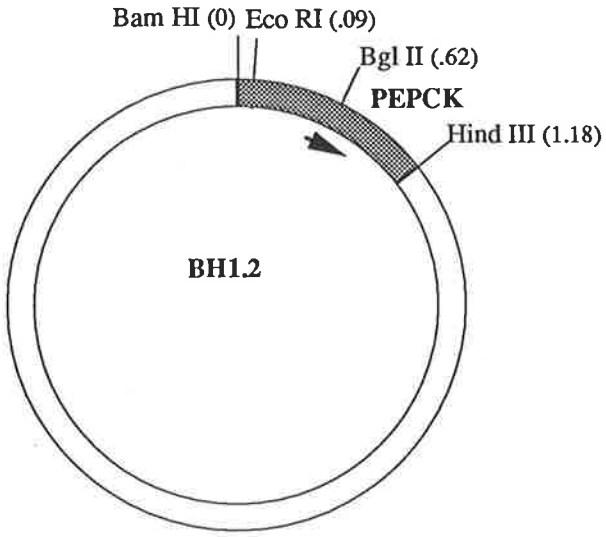
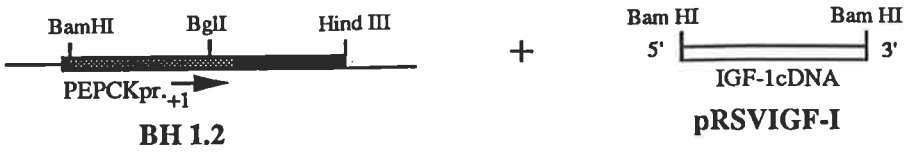
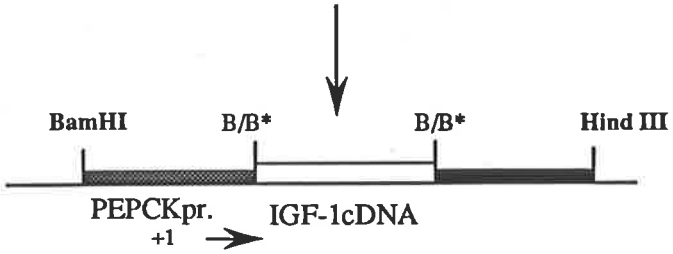

Bam HI (0)
Eco RI (.09)
Bgl II (.62)
PEPCK
Hind III (1.18)
BH1.2

FIGURE 3.2.2 Shows the steps involved in the production of the pPEPCK-IGF-Ia) construct. Briefly the IGF-I cDNA from pRSV-IGF-I was cloned into the Bgl II site of pBH1.2. After confirming the orientation of the IGF-I cDNA was correct with respect to the PEPCK promoter, the Bam HI/Hind III PEPCK-IGF-I gene was cloned into pBS and then SP 73 to gain useful restriction sites. Finally the PEPCK-IGF-I was cloned into pRSV-IGF-I replacing RSV-IGF-I as a Sal I/Bgl II fragment and positioning the PEPCK-IGF-I gene next to the hGH transcriptional termination signal.

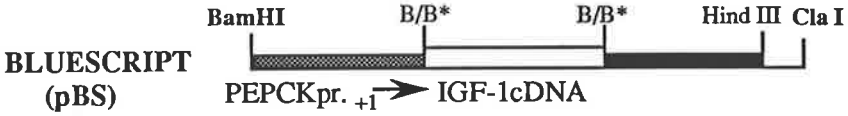
Construction of pPEPCKIGF-Ia



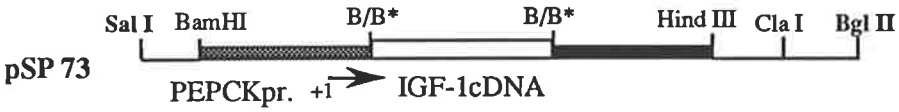
1. IGF-I cDNA cloned into Bgl II site of BH 1.2



2. Remove BamHI/HindIII fragment
ligate to bluescript Bam/Hind III



3. Remove BamHI/ClaI fragment
ligate to SP 73 Bam HI/ClaI



4. Remove Sal I/Bgl II fragment
ligate to pRSVIGF-1 minus
Sal/Bam fragment

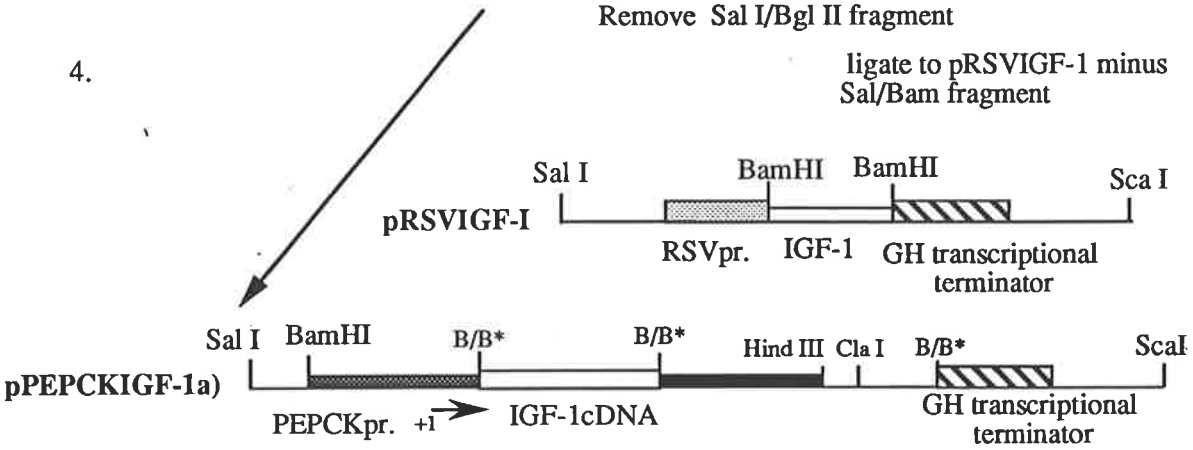


FIGURE 3.2.3 Subsequent to cloning of the hIGF-I cDNA Bam HI fragment into plasmid BH1.2, clones containing the 448 bp insert were sequenced using a 17 mer primer that binds within the IGF-I cDNA, to ensure that the correct orientation of the cDNA with respect to the PEPCK promoter had been attained. Shown is a small portion of sequence confirming that these 2 clones contained the desired orientation. The sequence may be read directly from the autoradiograph in a 5'-3' direction according to the arrow shown. Dideoxy sequencing was carried out with ^{32}P-dNTPs and the Klenow fragment of DNA Polymerase I (see materials and methods section 6.3.9) on double-stranded templates purified by gel filtration chromatography. The autoradiograph shown was exposed to film for 24 hours. The Bam HI/Bgl II site has no effect on translation as this region of the construct is transcribed but not translated

Sequencing to confirm orientation of IGF-I cDNA with respect to PEPCK promoter region

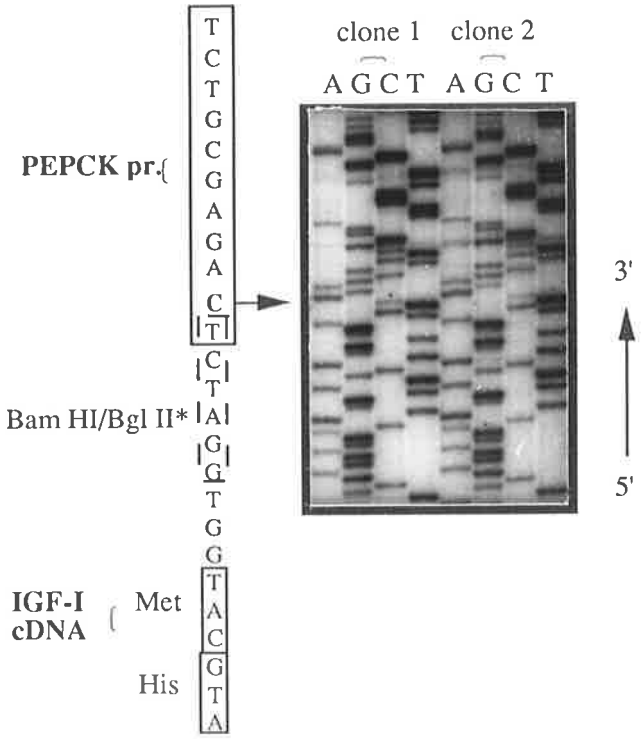
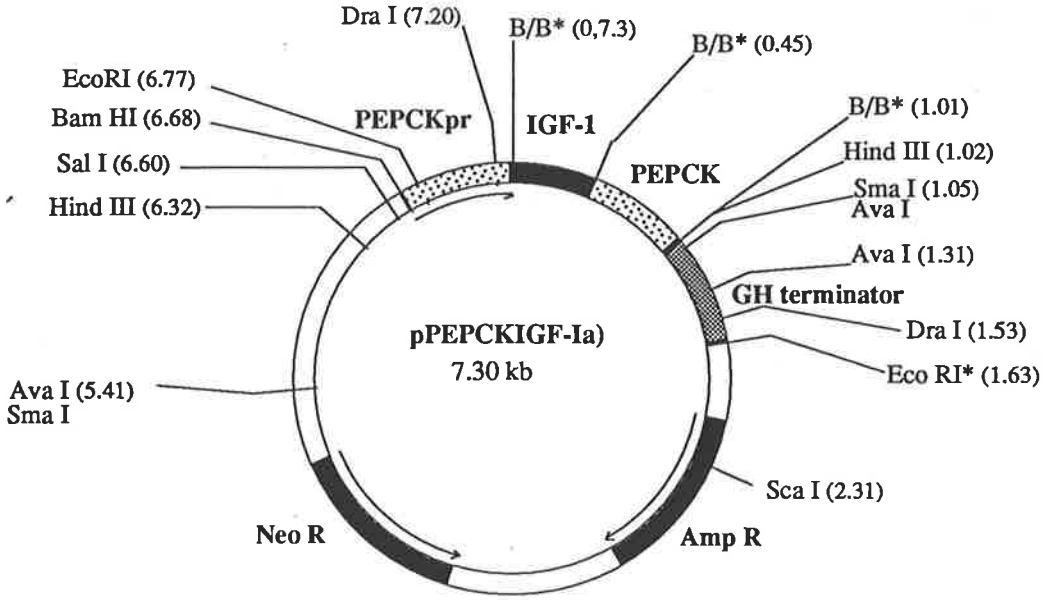


FIGURE 3.2.4 Shows a detailed map of plasmid pPEPCK-IGF-Ia). This 7.3 kb plasmid contains the rat PEPCK promoter linked to a hIGF-I cDNA and the hGH transcriptional termination signal. In addition the plasmid has the aminoglycoside phosphotransferase gene (Neo ^{R}) enabling selection of permanent cell-lines. For transgenesis the plasmid was digested with Sal I and Sca I and the 2.96 kb fragment purified for microinjection. The sites denoted B/B* represent sites of ligation between compatible Bam HI and Bgl II ends resulting in destruction of both sites. Eco RI* is a destroyed Eco RI site. Restriction sites relevant to discussion have been shown. A linear representation of the 2.96 kb Sal I/Sca I transgene fragment is given below with the sizes of its composite parts indicated.

Map of pPEPCK-IGF-Ia) plasmid



Sal I, Bam HI, Eco RI, Sca I- single sites
Hind III, Sma I, Dra I- two sites (Dra I sites outside of transgene are not shown)
Ava I-three sites
Eco RI*- destroyed site
B/B*-destroyed site

PEPCK-IGF-Ia) Transgene

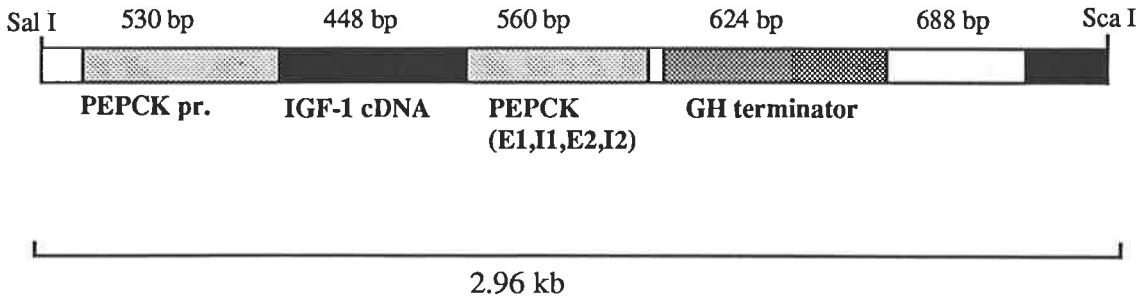


FIGURE 3.2.5 i) Plasmid pPEPCK-IGF-Ia) was digested with the following restriction enzymes and the digests analysed by electrophoresis on a 1 % TBE buffered Agarose gel containing 3mg/ml Ethidium Bromide. Eco RI digested SPP1 size markers were co-electrophoresed

lane 1: SPP1 markers

lane 2: Sal I

lane 3: Hind III

lane 4: Bam HI

lane 5: Eco RI

lane 6: Hind III / Bam HI

lane 7: Eco RI / Sca I

lane 8: Sal I / Sca I

lane 9: Ava I

lane 10: Dra I

ii) Additional digests were carried out on pPEPCK-IGF-I a) (as below) and analysed on a 1 % TBE bufferd Agarose gel. The gel was subsequently transferred to Nytran™ and the DNA crosslinked to the filter with UV light. The filter was hybridised at 42 °C overnight in the presence of the oligolabelled Bam HI / Bgl II fragment of the PEPCK promoter. After washing the filter at high stringency in 0.1 x SSC/ 0.1 % SDS at 65 °C for 1 hour, autoradiography was carried out for 4 hours at -80 °C.

lane 1: Sal I

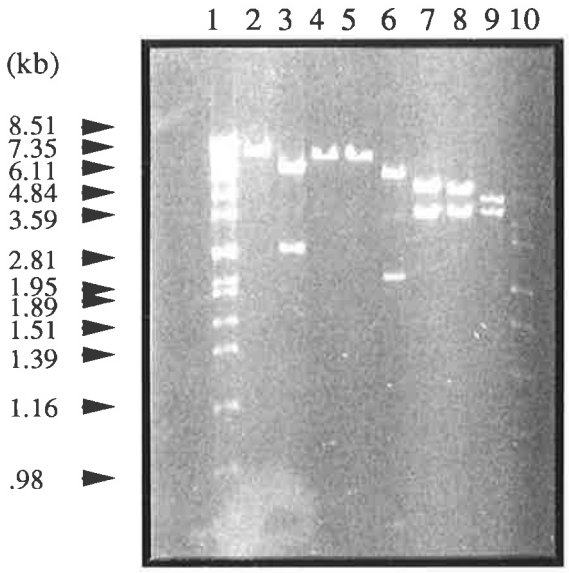
lane 2: Sal I / Sca I

lane 3: Hind III

lane 4: Ava I

lane 5: Dra I

i)


1 2 3 4 5 6 7 8 9 10
(kb)
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.89
1.51
1.39
1.16
.98

ii)

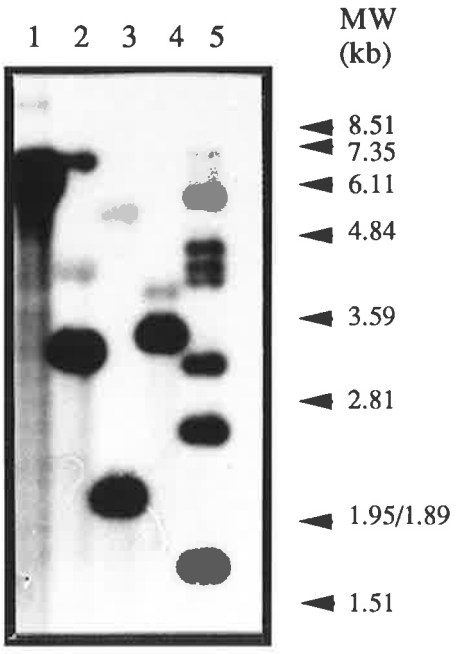

1 2 3 4 5
MW
(kb)
8.51
7.35
6.11
4.84
3.59
2.81
1.95/1.89
1.51

FIGURE 3.2.6 Dideoxy sequencing (section 6.3.9) of clones following site-directed mutagenesis to remove the three N-terminal amino acids from the IGF-I molecule. Mutagenesis (section 6.3.12) was carried out on PEPCK-IGF-I templates cloned into the pBS vector. Sequencing was performed on double-stranded templates purified by gel filtration chromatography. A clone containing the non-mutated IGF-I sequence is shown (left) along side a mutagenic clone (right). The sequence shown can be read directly from the autoradiograph. The emboldened **T** residue is common to both sequences and the * represents the point of mutagenesis in the PEPCK-des(1-3)IGF-I clone. (Please note the order of the G and C lanes differs between each clone)

5' GCA GAG CGT CTC CGG TCC AGC 3' (sequence off gel)

CGT CTC GCA GAG GCC AGG TCG (opposite strand)

Cys Leu Thr **Glu^{3} Pro^{2} Gly^{1}** Ala (coding capacity of compliment)

Sequencing to confirm des(1-3)IGF-I mutation

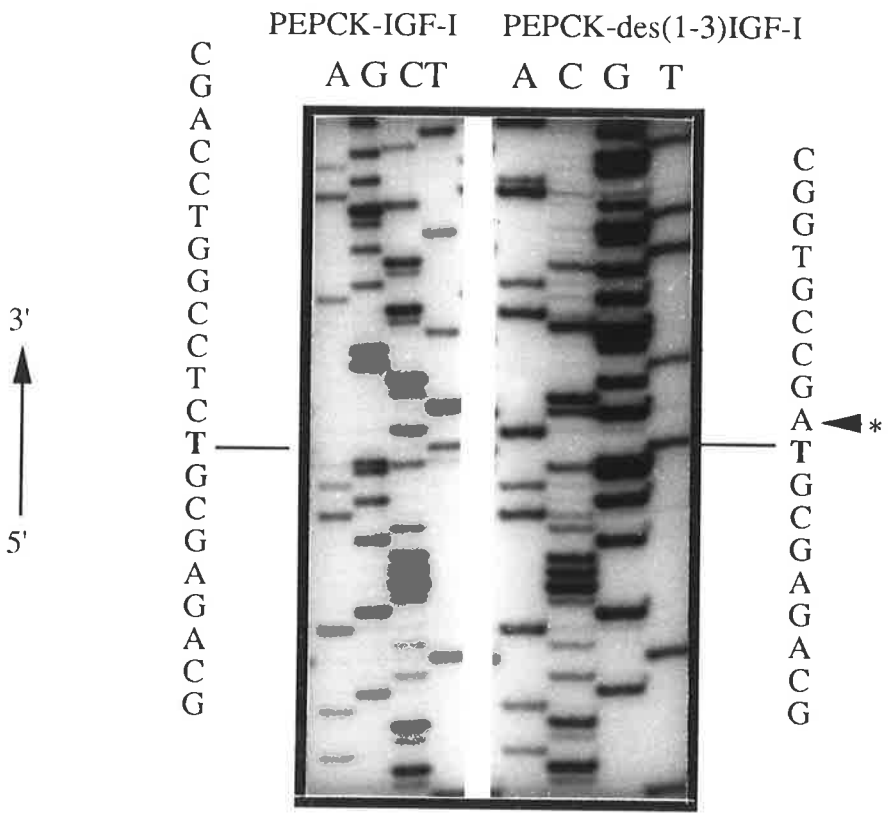
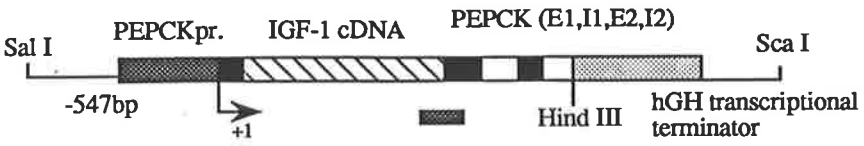
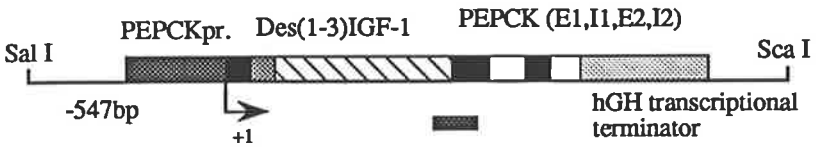


FIGURE 3.2.7 Shown are the four constructs produced during this study. The small box below the PEPCK-IGF-I a) construct illustrates the position of a 30 mer oligonucleotide primer, designed to create a Hind III site and facilitate removal of the 3' PEPCK exon 1, intron 1, exon 2 and intron 2 (E1,I1,E2,I2) sequences.

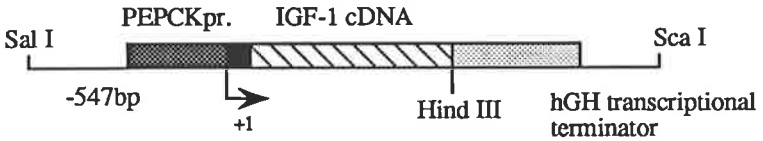
1) **PEPCK -IGF-1a)**



2) **PEPCK -3N IGF-1a)**



3) **PEPCK -IGF-1b)** (**lacks PEPCK introns**)



4) **PEPCK -3N IGF-1b)** (**lacks PEPCK introns**)

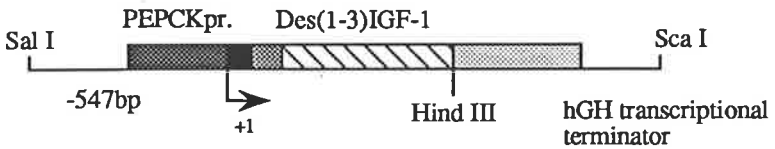


FIGURE 3.2.8 The sequence of the coding strand of the hIGF-I cDNA and GH transcriptional terminator is shown as it appears in pRSVIGF-I (figure 3.2.1). The Bam HI, Rsa I and Sma I sites of the IGF-I cDNA are shown in bold typeface. The position of the 17 mer oligonucleotide primer used throughout this study is indicated.

Sequence of IGF-I /hGH region of PEPCK-IGF-I transgene

met his thr met ser ser ser his leu phe tyr leu ala leu cys leu leu thr
TCCACCATGCACACCATGTCCTCCTCGCATCTCTTCTACCTGGCGCTGTGCCTGCTCACC

phe thr ser ser ala thr ala gly pro glu thr leu cys gly ala glu leu val asp ala
TTCACCAGCTCTGCCACGGCTGGACCGGAGACGCTCTGCGGGGCTGAGCTGGTGGATGCT

leu gln phe val cys gly asp arg gly phe tyr phe asn lys pro thr gly tyr gly ser
CTTCAGTTCGTGTGTGGAGACAGGGGCTTTTATTTCAACAAGCCCACAGGGTATGGCTCC

hIGF-I cDNA (448 bp)

17mer

ser ser arg arg ala pro gln thr gly ile val asp glu cys cys phe arg ser cys asp
AGCAGTCGGAGGGCGCCTCAGACAGGTATCGTGGATGAGTGCTGCTTCCGGAGCTGTGAT

leu arg arg leu glu met tyr cys ala pro leu lys pro ala lys ser ala arg ser val
CTAAGGAGGCTGGAGATGTATTCGGCACCCCTCAAGCCTGCCAAGTCAGCTCGCTCTGTC

arg ala gln arg his thr asp met pro lys thr gln lys glu val his leu lys asn ala
CGTGCCCAGCGCCACACCGACATGCCCAAGACCCAGAAGGAA**GTAC**ATTTGAAGAACGCA

Rsa I

ser arg gly ser ala gly asn lys asn tyr arg met
AGTAGAGGGAGTGCAGGAAACAAGAACTACAGGATGTAGGAAGACCCTCCTGAGGAGTGA

Bam HI Sma I

AGAGTGACATGCCACCGCA**GGATCC**CCGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCT

CTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAATTAAGTT

GCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGTGGTAT

GGAGCAAGGGGCCCAAGTTGGGAAGACAACCTGTAGGGCCTGCGGGGTCTATTCGGGAAC

CAAGCTGGAGTGCAGTGGCACAATCTTGGCTCACTGCAATCTCCGCCTCCTGGGTTCAAG

CGATTCTCCTGCCTCAGCCTCCCGAGTTGTTGGGATTCCAGGCATGCATGACCAGGCTCA

GCTAATTTTTGTTTTTTTGGTAGAGACGGGGTTTCACCATATTGGCCAGGCTGGTCTCCA

ACTCCTAATCTCAGGTGATCTACCCACCTTGGCCTCCCAAATTGCTGGGATTACAGGCGT

GAACCACTGCTCCCTTCCCTGTCCTTCTGATTTTAAAATAACTATACCAGCAGGAGGACG

TCCAGACACAGCATAGGCTACCTGCCATGGCCCAACCGGTGGGACATTTGAGTTGCTTGC

TTGGCACTGTCCTCTACTGCGTTGGGTCCACTCAGTAGATGCCTGTTGAATTC

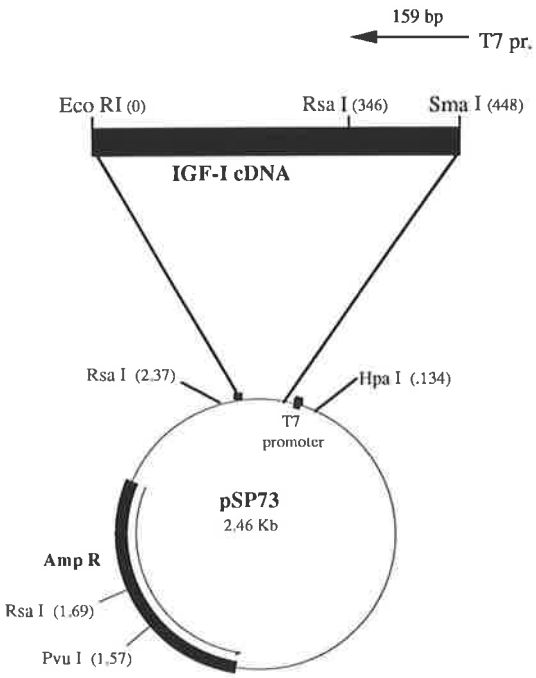
hGH transcriptional termination sequence (624 bp)

FIGURE 3.2.9 i) A schematic representation of the riboprobe used to detect hIGF-I mRNA. An Eco RI / Sma I fragment containing the entire hIGF-I cDNA was cloned directionally into the polylinker of pSP73. The template for transcription was prepared by gel filtration chromatography after first being digested to completion with Rsa I.

ii) Transcription with T7 polymerase gives rise to a full-length probe of 159 bp. Twenty micrograms of RNA isolated from either yeast (lane 2), a hIGF-I expressing CHO cell-line (McKinnon et., al 1993) (lane 3), mouse liver (lane 4) or non-transfected CHO cells (lane 5) was analysed by ribonuclease protection assay using an Ambion RPA kit. After overnight hybridisation at 55 ^{o}C the hybrids were digested with RNA se A+T1 (except lane 2) and were resolved on a 6 % polacrylamide gel. Autoradiography was carried overnight at -80 ^{o}C.

Construction of pSP73-IGF-I

i)



ii)

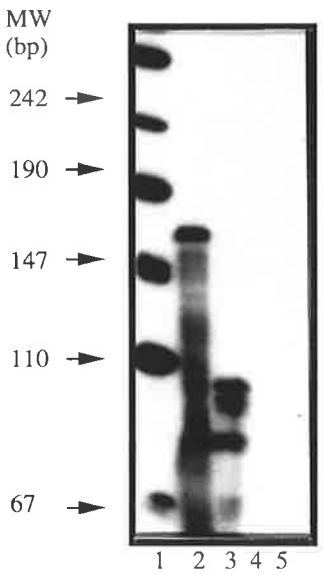


FIGURE 3.2.10 RNA was harvested from individual G418 resistant CHO clones transfected with either the RSV-IGF-I or PEPCK-IGF-Ia) constructs, after 24 hr. incubation in serum-free medium containing dexamethasone (50 nM) and was subject to analysis by ribonuclease protection. RNA (20 μg) was hybridised overnight at 55 °C with the 159 bp hIGF-I riboprobe (except where stated). Following digestion with RNase A+T1, the hybrids were resolved on 6 % polyacrylamide gels before autoradiography for 36 hours. The sizes of radiolabelled Hpa II digested pUC DNA markers co-electrophoresed on the gel are shown on the right. The samples were as follows

lane 1: yeast RNA (20 μg)

lane 2: RSV-IGF-I CHO clone (20 μg)

lane 3: mouse liver RNA + B Actin riboprobe (protects a 250 bp species)

lane 4: mouse liver RNA +hIGF-I riboprobe

lane 5: RSV-IGF-I CHO clone A (20 μg)

lane 6: PEPCK-IGF-Ia) clone 1C

lane 7: PEPCK-IGF-Ia) clone 1D

lane 8: PEPCK-IGF-Ia) clone 1K

lane 9: PEPCK-IGF-Ia) clone 1L

lane 10: PEPCK-IGF-Ia) clone 1M

lane 11: PEPCK-IGF-Ia) clone 1N

lane 12: PEPCK-IGF-Ia) clone 1O

Expression of hIGF-I mRNA in permanent CHO cell-lines

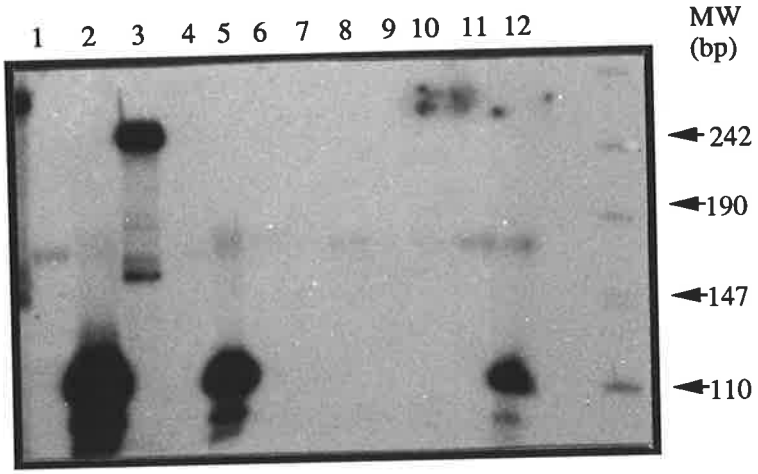


FIGURE 3.2.11 RNA (25 μg) was harvested from clones transfected with either the PEPCK-IGF-Ia), PEPCK-IGF-I b), PEPCK-Des(1-3)IGF-I a) or PEPCK-Des(1-3)IGF-I b) constructs and grown overnight in serum-free medium containing dexamethasone (50 nM). RNA was then analysed by ribonuclease protection using the 159 bp hIGF-I riboprobe. Hybridisation was overnight at 55 ^{o}C , before the hybrids were digested with RNase A+T1. Digestion was incomplete in this assay as shown by the large amount of free probe (159 bp) present in most lanes. Hybrids were resolved on 6 % polyacrylamide gels.

i) Shows RNA samples from CHO transfectants.

A= PEPCK-IGF-Ia) clones

B= PEPCK-IGF-Ib) clones

C= PEPCK-Des(1-3)IGF-I clones

D= PEPCK-Des(1-3)IGF-I clones

E=non-transfected CHO cells.

These autoradiographs were exposed for 4 days

ii) Shows samples from H4IIE transfectants

F= PEPCK-IGF-Ia) clones

G= PEPCK-IGF-Ib) clones

H= PEPCK-Des(1-3)IGF-I clones

I= PEPCK-Des(1-3)IGF-I clones

J= non-transfected H4IIE cells

These autoradiographs were exposed for 7 days .

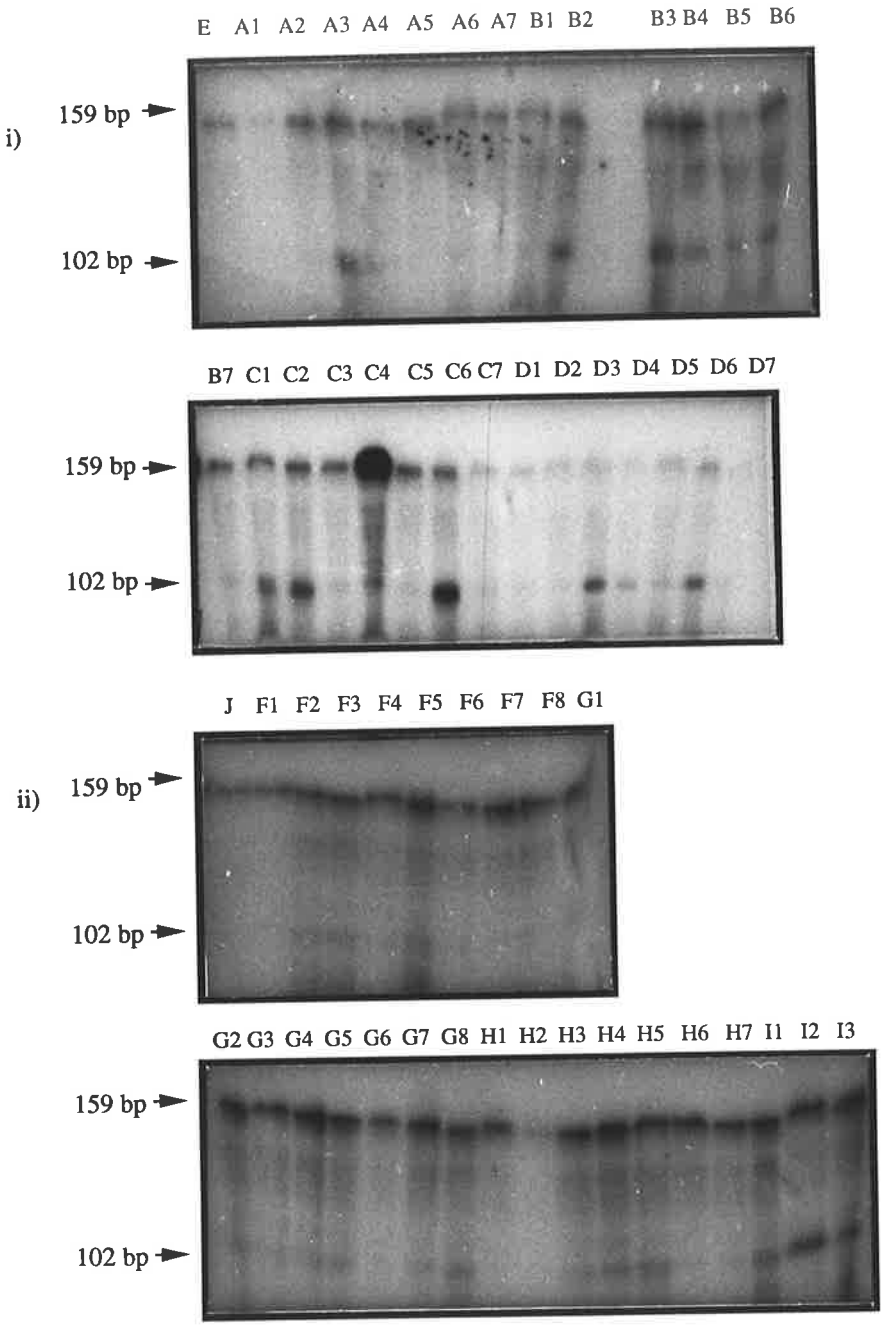

i)
E A1 A2 A3 A4 A5 A6 A7 B1 B2 B3 B4 B5 B6
159 bp
102 bp
B7 C1 C2 C3 C4 C5 C6 C7 D1 D2 D3 D4 D5 D6 D7
159 bp
102 bp
ii)
J F1 F2 F3 F4 F5 F6 F7 F8 G1
159 bp
102 bp
G2 G3 G4 G5 G6 G7 G8 H1 H2 H3 H4 H5 H6 H7 I1 I2 I3
159 bp
102 bp

FIGURE 3.2.12 i) Dot-blot analysis was carried out on transfected CHO and H4IIE clones. DNA (5 μg) was denatured and applied to Nytran™ membrane under vacum before the filter was cross-linked with UV light. After prehybridisation, the filter was probed overnight at 42 °C with the 487 bp Sma I/Dra I fragment of the hGH transcriptional terminator (see figure3.2.4) and washed at high stringency (0.1 x SSC/ 0.1 % SDS at 65 °C for 45 min.) before autoradiography for 24 hours. In addition to test samples, a positive copy number control series was applied containing pPECK-IGF-I a) diluted in mouse genomic DNA (5 μg). The bracketed numbers in row **a** indicate the equivalent copy number of this series. A key to sample identity is given below:

A=CHO PEPCK-IGF-Ia), B= CHO PEPCK-IGF-I b), C= CHO PEPCK-Des(1-3)IGF-I D=CHO PEPCK-Des(1-3)IGF-I, E=non-transfected CHO, F= H4IIE PEPCK-IGF-Ia) G= H4IIE PEPCK-IGF-Ib), H=H4IIE PEPCK-Des(1-3)IGF-I, I=H4IIE PEPCK-Des(1-3)IGF-I

J= non-transfected H4IIE.

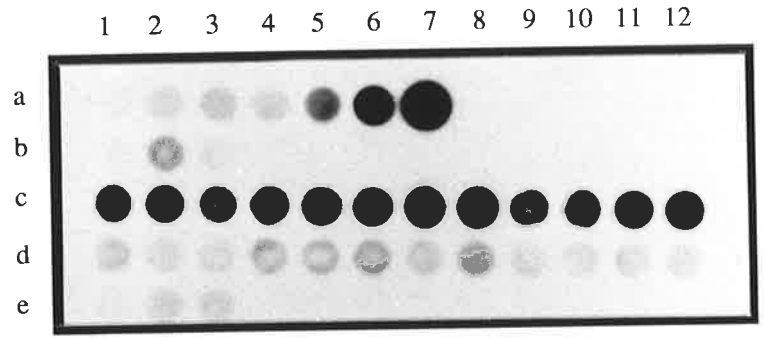
	1	2	3	4	5	6	7	8	9	10	11	12
a	(1)	(2)	(4)	(8)	(24)	(32)	(40)					
b	mouse genomic	E	J									
c	A2	A6	A7	B1	B2	B5	C1	C6	C7	D1	D4	D6
d	F2	F3	F4	F5	G3	G7	G8	H1	H2	H3	H4	H5
e	I1	I4	I5									

ii) Polymerase Chain Reaction was carried out as described in section 6.3.13 ii), on DNA isolated from CHO and H4IIE transfectants. The primers mapped to the PEPCK promoter region and IGF-I cDNA respectively. Aliquots of each reaction (5 ul) were electrophoresed on 2 % TBE buffered Agarose gels containing Ethidium Bromide (3 mg/ml) The 285 bp product was visualised by UV irradiation of the gels. The sizes of low molecular weight Hpa II digested pUC DNA markers are indicated by arrows to the left of the gel.

lane 1: Hpa II pUC markers
lane 2: no DNA control
lane 3: non-transfected CHO
lane 4: non-transfected H4IIE
lane 5: A6
lane 6: A7
lane 7: B2
lane 8: C1
lane 9: D1
lane 10: D4
lane 11: F3
lane 12: G3
lane 13: H4
lane 14: I1
lane 15: I4
lane 16: Hpa II Mkrs.

Dot-blot of permanent CHO and H4IIE cell-lines

i)



PCR analysis of permanent CHO and H4IIE cell-lines

ii)

FIGURE 3.2.13 Conditioned media from several PEPCK-IGF-I or PEPCK-Des(1-3)IGF-I CHO clones was collected after overnight incubation in the presence of serum-free medium and dexamethasone (50 nM). These samples (20 µl of 8-fold concentrated) were then tested for their ability to stimulate incorporation of ^{3}H-Leucine into total cell protein of semi-confluent rat L6 myoblasts. Included in the assay was a series of hIGF-I standards and 10 % FBS as positive controls. The negative controls were PBS (MEM) and conditioned media from non-transfected CHO cells (E). The results are expressed as the % stimulation above control protein synthesis observed in the presence of PBS alone. Values for clones A1, C7, D1, D4, D6 and E were the means of triplicate assays whereas values for A2, C2, C6 and D3 were means of duplicates.

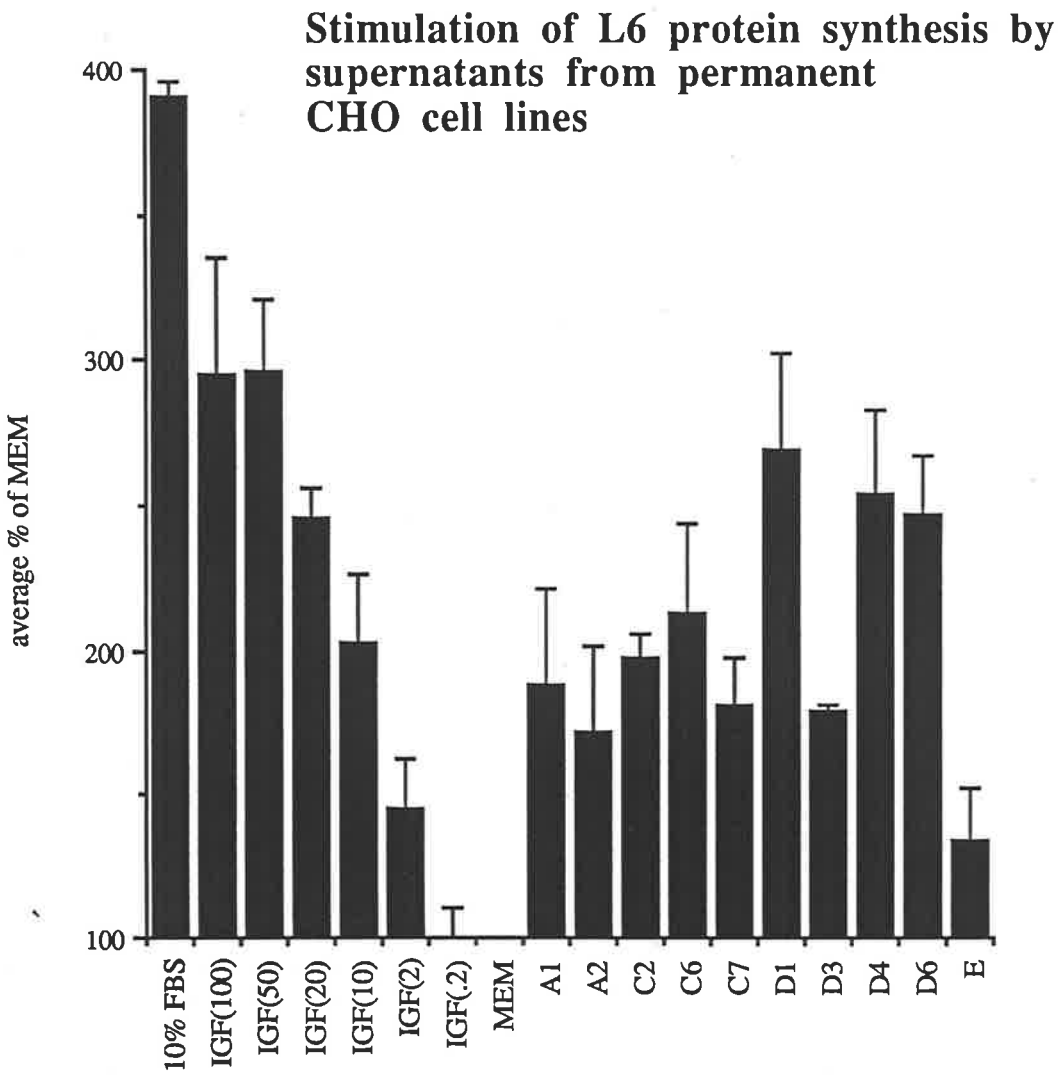
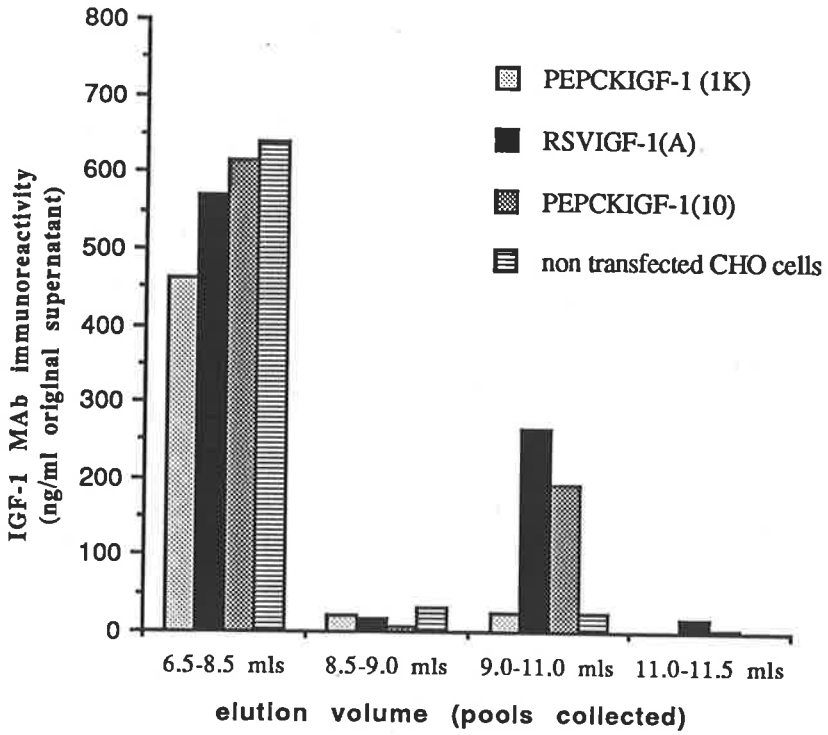

Stimulation of L6 protein synthesis by supernatants from permanent CHO cell lines
average % of MEM
400
300
200
100
10% FBS
IGF(100)
IGF(50)
IGF(20)
IGF(10)
IGF(2)
IGF(.2)
MEM
A1
A2
C2
C6
C7
D1
D3
D4
D6
E

FIGURE 3.2.14 Media conditioned by permanently transfected CHO cells (RSV-IGF-I (clone A), or PEPCK-IGF-I (clones 1O and K)) or non-transfected CHO cells grown overnight in the presence of dexamethasone (50 nM), was FREON extracted and chromatographed by gel filtration under acid conditions. Four pools of eluent were collected and aliquots (50 µl) of each fraction assayed in triplicate in a radioimmunoassay employing a monoclonal antibody to hIGF-I (section 6.3.20). The position of elution of authentic hIGF-I,10.4 ml, was confirmed by chromatography of ^{125}-[I]-IGF-I before and after the samples.

FIGURE 3.2.15 Conditioned medium from CHO clones containing RSV-IGF-I (RSV) , PEPCK-IGF-Ia) (PCK) or from non-transfected cells (CHO) was collected after overnight incubation in the presence of serum-free medium and dexamethasone (50 nM). Each sample was then concentrated four-fold and 50 µl assayed for its ability to stimulate protein synthesis by way of ^{3}-H-Leucine incorporation into rat L6 myoblasts. Triplicate determinations for each supernatant were performed and expressed as the % stimulation achieved in the presence of PBS (MEM) only. A series of hIGF-I standards were included as positive controls along with 10 % FBS.

Gel filtration and MAb RIA of CHO supernatants



L6 protein synthesis activity of permanent CHO cell lines

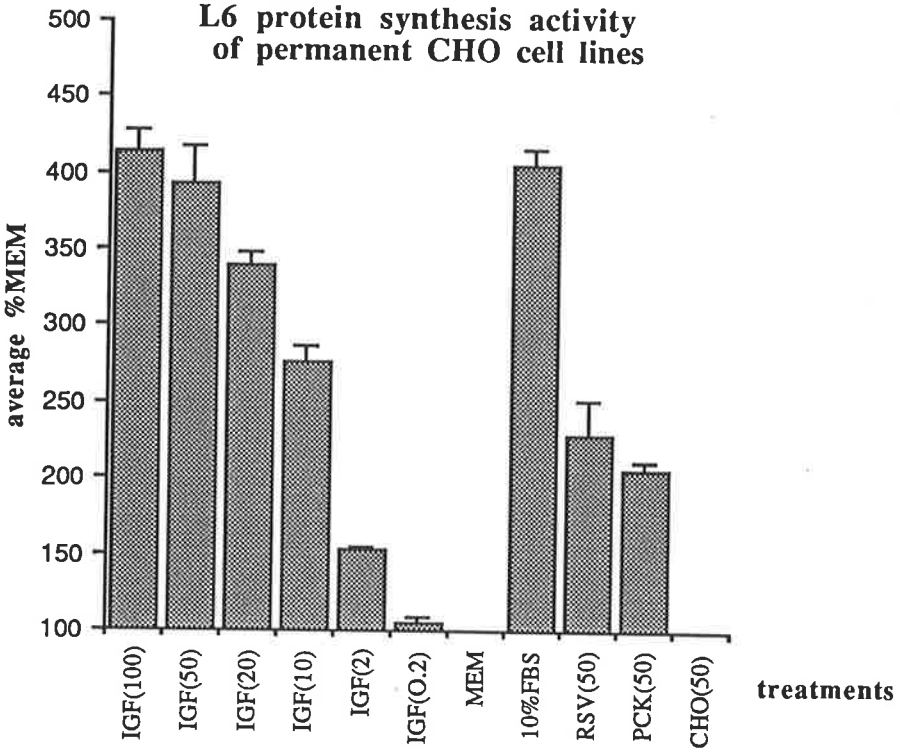
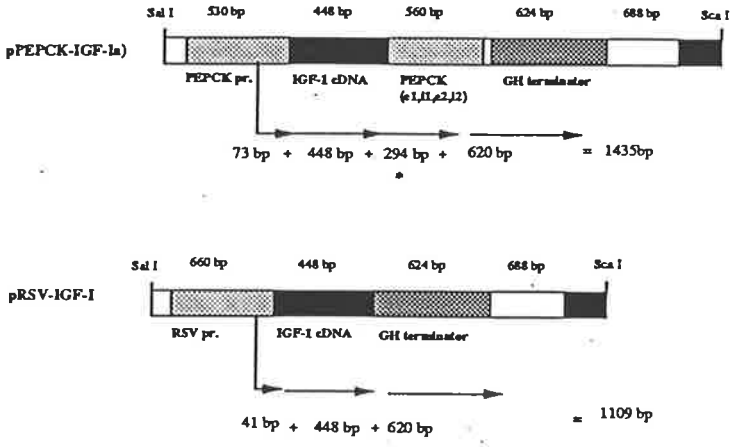


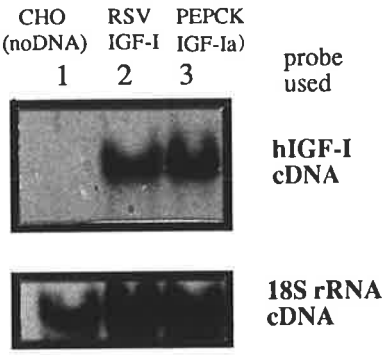
FIGURE 3.2.16 **i)** Northern analysis was performed on RNA (20 μg) isolated from RSV-IGF-I CHO clone A, PEPCK-IGF-Ia) clone 1O and non-transfected CHO cells. RNA was isolated after overnight incubation in serum-free media and run on a 1% Phosphate-buffered Agarose gel containing formaldehyde (10 %). The gel was subsequently transferred to Nytran™ and RNA cross-linked to the filter with UV light. The filter was initially probed with the 448 bp Bam HI hIGF-I cDNA and exposed for one week after washing the filter at high stringency in 0.1 x SSPE/ 0.1 % SDS at 60 °C for 30 minutes. Loadings were checked by reprobing the filter with a rat 18 S rRNA probe and autoradiography for 4 hours.



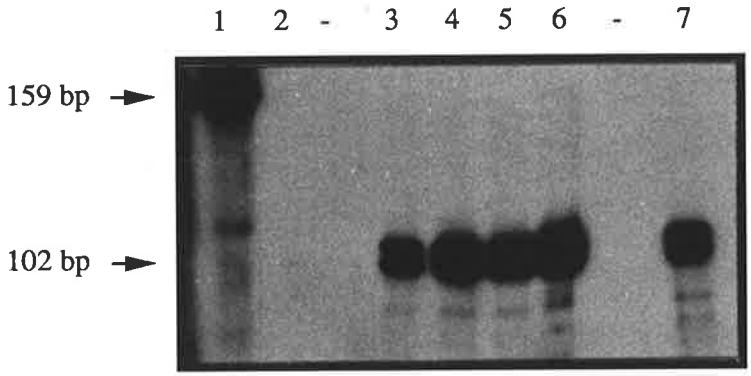
• It was not known whether these PEPCK exonic sequences would be included or spliced out of the mature cytoplasmic mRNA species.

ii) CHO clone 1O containing the PEPCK-IGF-Ia) construct was grown overnight in the presence of serum-free (F12) medium (lane 4), F12+ 10 % FCS (lane 3), F12+ 100 nM dexamethasone (lane 5), F12+ 500 nM dexamethasone (lane 6) or F12 + 100 nM dexamethasone + insulin (1 ng/ml) (lane 7). RNA was harvested and ribonuclease protection carried out using 25 μg of total RNA and the 159 bp hIGF-I riboprobe. After digestion, the protected species were resolved on 6 % polyacrylamide gels and autoradiography carried out for 48 hours. Only the protected 102 bp species and mature probe (lane 1) are shown with arrows. Lane 2 contained yeast RNA as a negative control. Densitometry was performed on the 102 bp species and is illustrated by the bar graph below.

i)


CHO
(noDNA)
RSV
IGF-I
PEPCK
IGF-Ia)
1
2
3
probe
used
hIGF-I
cDNA
18S rRNA
cDNA

ii)


1 2 - 3 4 5 6 - 7
159 bp
102 bp

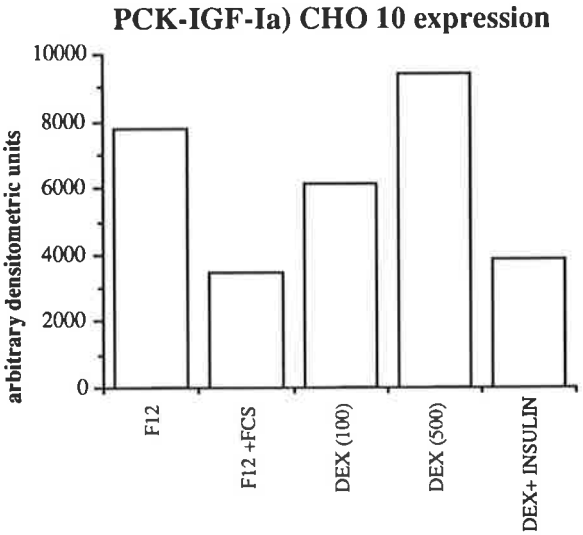
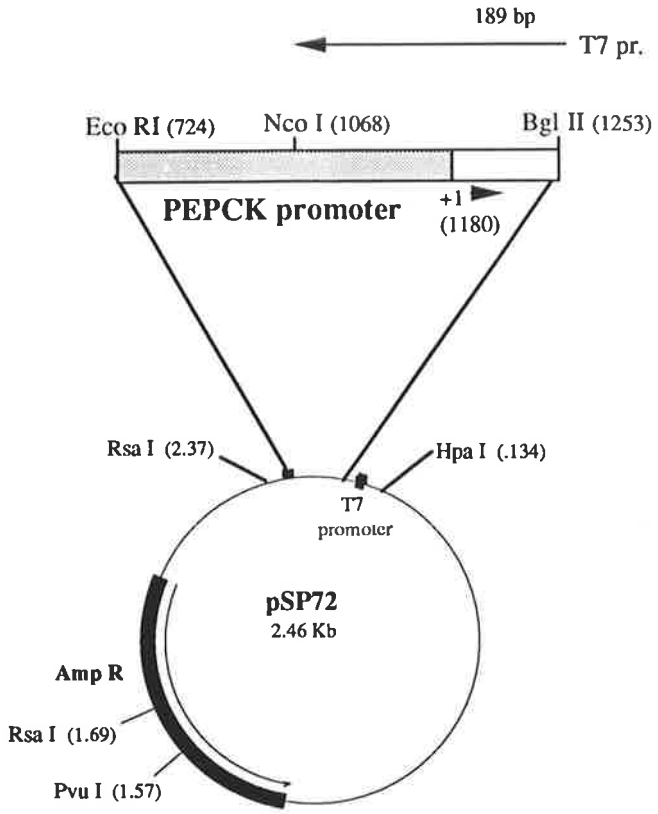

PCK-IGF-Ia) CHO 10 expression
arbitrary densitometric units
10000
8000
6000
4000
2000
0
F12
F12 +FCS
DEX (100)
DEX (500)
DEX+ INSULIN

FIGURE 3.2.17 **i)** To produce a suitable probe for analysis of PEPCK mRNA, an Eco RI / Bgl II fragment containing the transcription initiation site of the PEPCK gene was isolated and directionally cloned into the ploylinker of pSP72. The construct was linearised with Nco I prior to transcription of antisense mRNA using T7 polymerase. Transcription produces a 189 bp riboprobe which should protect a 70-73 bp region of PEPCK mRNA.

ii) Total RNA was harvested from confluent cultures of rat Reuber H4IIE Hepatoma cells grown overnight in serum-free DME medium (lane 2), DME + dexamethasone (100 nM) (lane 3) , DME + insulin (1 ng/ml) (lane 4), DME + IGF-I (5 ng/ml) (lane 5), DME + IGF-I (10 ng/ml)(lane 6) or DME + IGF-I (50 ng/ml) (lane 7). Ribonuclease protection was carried out on 25 μg of RNA from each culture using the 189 bp PEPCK riboprobe (shown above). The protected 70-75 bp species are shown with an arrow. These species were detected in 25 μg of total RNA isolated from rat liver (lane 8), but were not detected in the negative control, yeast RNA (lane 1). Densitometry was carried out on the protected species and is illustrated graphically below

i)


189 bp
T7 pr.
Eco RI (724)
Nco I (1068)
Bgl II (1253)
PEPCK promoter
+1
(1180)
Rsa I (2.37)
Hpa I (.134)
T7
promoter
pSP72
2.46 Kb
Amp R
Rsa I (1.69)
Pvu I (1.57)

ii)

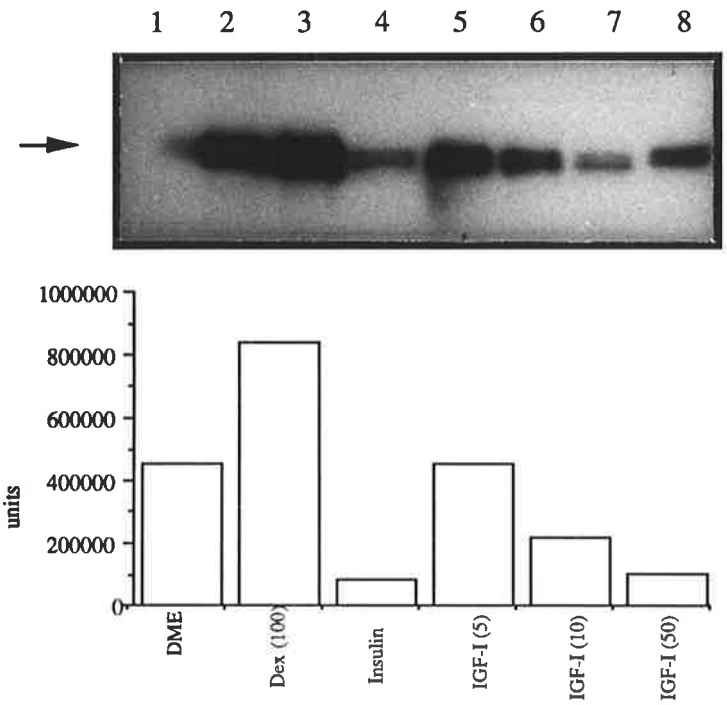
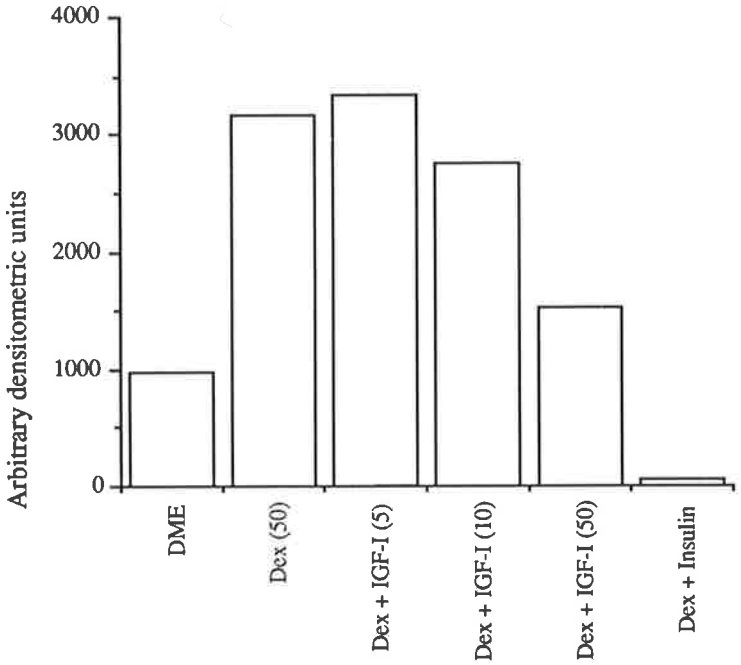

1 2 3 4 5 6 7 8
Arbitrary densitometric units
1000000
800000
600000
400000
200000
0
DME
Dex (100)
Insulin
IGF-I (5)
IGF-I (10)
IGF-I (50)

FIGURE 3.2.18 Ribonuclease protection analysis was performed on 25 μg of total RNA isolated from confluent H4IIE cultures using the 189 bp PEPCK riboprobe. Cells were grown overnight in the presence of serum-free DME medium (lane 1), DME + dexamethasone (50 nM) (lane 2), DME + dexamethasone (50 nM) + IGF-I (5 ng/ml) (lane 3), DME + dexamethasone (50 nM) + IGF-I (10 ng/ml) (lane 4), DME+ dexamethasone (50 nM) + IGF-I (50 ng/ml) (lane 5) or DME + dexamethasone (50 nM) + Insulin (1 ng/ml) (lane 6). Only the protected 70-73 bp species are shown. Quantitation of the autoradiograph by densitometry is presented as a bar graph below.


1 - - - 2 3 4 5 6


Arbitrary densitometric units
4000
3000
2000
1000
0
DME
Dex (50)
Dex + IGF-I (5)
Dex + IGF-I (10)
Dex + IGF-I (50)
Dex + Insulin

3.3 DISCUSSION:

The major aim of the work presented in this chapter was to produce a construct linking the PEPCK promoter to the hIGF-I cDNA that would express biologically active hIGF-I *in vitro,* as a prelude to transgenesis studies. Hence, for its end use *in vivo* , intronic sequences from the PEPCK gene were included in the construct.

Intronless copies of protein encoding genes are often expressed poorly compared to genomic constructs. In some cases this may be due to the presence within such introns of *cis* acting elements (eg. enhancers) that can influence transcription initiation or elongation (Gillies et al., 1983; Mitchell et al., 1989). However, in other cases no such sequences have been observed and it is thought that introns may influence some aspects of nucleosome composition or binding thereby facilitating access to the chromosome by transcription factors (Sveren et al., 1990). Additionally, introns may aid in RNA stability and/or transport by beneficial association with the splicing machinery of the cell.(Dreyfuss et al., 1988; Buchman et al., 1988; Nesic et al., 1993). Although effects of introns on gene expression *in vitro* have been reported, several studies have observed pronounced effects of introns on expression *in vivo* without accompanying changes to *in vitro* expression (Brinster et al., 1988; Palmiter et al., 1991).

Because of the low levels of expression observed in my permanently transfected cell lines it was not possible to strictly examine the relationship between expression levels and the presence or absence of introns in the constructs. However, examination of transfected CHO cells by ribonuclease protection showed that clones containing constructs with or without introns were able to produce mRNA, indicating no absolute dependence on introns for expression of these constructs *in vitro*. This is not surprising since previous expression of hIGF-I cDNAs has been achieved in CHO cells using intronless constructs (McKinnon et al., 1990; Bovenberg et al., 1990). Whether the levels of IGF-I expression obtained could be increased by including introns in the constructs is still unanswered.

Transfection of pPEPCK-IGF-I a) into CHO cells led to the recovery of a large number of positive clones. Although all isolated clones expressed the neomycin resistance gene, only low level expression of hIGF-I was observed in most, as judged by ribonuclease protection. Of relevance to this observation, it has previously been reported that selection for one marker(eg. antibiotic resistance) may result in a skewed population in which expression from a non-selected promoter is suppressed (Engelhardt et al., 1990).

Alternatively, the low level expression of hIGF-I may be due to these cells lacking liver-specific trans-acting factors required by the PEPCK promoter for efficient expression. The exceptional levels of expression observed in one particular clone (1O) may therefore reflect favourable integration of the construct into the CHO genome, resulting in an enhancement of expression from the PEPCK promoter due to *cis* acting elements flanking the site of insertion. Despite the higher levels of expression observed for this particular clone, production of hIGF-I mRNA was regulated by dexamethasone and insulin suggesting that transcription was initiated from the PEPCK promoter. The amount of hIGF-I secreted by this clone (190 ng/ml) was low compared to production by clones where the IGF-I gene was driven by the RSV LTR promoter (McKinnon et al., 1990) or the Drosophila hsp 70 Heat shock promoter (Bovenberg et al., 1990). These studies were designed to produce significant quantities of cell-derived hIGF-I whereas my aim was merely to establish that regulatable expression was achievable.

Because CHO cells were not highly gluconeogenic a better cell-line in which to study regulation of the constructs and therefore the transgenes was sought. The liver was the desired major source of expression for these transgenes *in vivo* due to it also being the predominant source of endocrine IGF-I in the rat (Phillips et al., 1976). Hence, rat Reuber H4IIE cells were chosen as a suitable cell-line for transfection. This cell-line had been extensively used to study the regulation of the rat PEPCK gene (Sasaki et al., 1984; Magnuson et al., 1987). However, expression in the G418 resistant H4IIE clones was extremely poor for each construct. To explain this result, I was able to show that not only could hIGF-I suppress the endogenous levels of PEPCK mRNA in these cells but it could do so in the presence of a known inducer of PEPCK transcription (dexamethasone). Since it was known that the 560 bp of PEPCK promoter used in my constructs contained sequences responsible for mediating the glucocorticoid, cAMP and insulin effects on the rat PEPCK gene (Roesler et al., 1989; Liu et al., 1991), it was likely that hIGF-I could also inhibit transcription from these constructs. Thus, a feedback loop was probably in operation in the permanently transfected cell-lines, preventing significant levels of PEPCK mediated hIGF-I secretion.

The 2-3 fold induction of endogenous PEPCK mRNA levels by dexamethasone in the H4IIE cell-line found in my experiments after 24 hours of treatment was slightly lower than the 4-fold induction previously observed in this cell-line after treatment with 500 nM dexamethasone but was consistent with the induction of transcription previously reported in

this cell-line by 50 nM of dexamethasone (Granner et al., 1985). Also this dexamethasone induction of PEPCK mRNA is within the range reported by Peterson et al., (1989) after 24 hours of treatment. Similarly, insulin's ability to"dominantly" inhibit production of PEPCK mRNA in H4IIE cells even in the presence of dexamethasone (as shown in this chapter) and other PEPCK inducers has been described previously (Granner et al., 1983; Magnuson et al., 1987).

No studies had previously been carried out to examine the effect of hIGF-I on PEPCK expression in H4IIE cells. The 50-100 fold lower potency of hIGF-I in suppressing PEPCK mRNA levels I observed in these cells compared to insulin corresponds to the cross-reactivity of this peptide with the insulin receptor. Furthermore, H4IIEs are believed to possess only insulin receptors (Massague et al., 1982; Rechler et al., 1985) Together these findings suggest that hIGF-I is able to inhibit PEPCK mRNA expression by acting via the insulin receptor in an analogous fashion to insulin.

Although des(1-3)IGF-I was not tested directly for its ability to inhibit expression of PEPCK mRNA in my studies, this analogue has recently been shown to have increased affinity for the insulin receptors on H35B hepatoma cells (Francis et al., 1993 (in press)). Thus it seems likely that lack of expression observed for H4IIE clones containing the des(1-3)IGF-I constructs is also explicable by a strong feedback inhibition of the PEPCK promoter by secreted des(1-3)IGF-I acting via the insulin receptor.

To more fully assess this proposition it is necessary to evaluate the regulation of IGFBPs in this cell-line. The major binding protein species produced by H4IIE cells has been shown to be IGFBP-1 (Unterman et al., 1990). Interestingly, dexamethasone has been shown to rapidly stimulate the production of IGFBP-1 in H4IIE cells (Orlowski et al., 1990) as has cAMP (Unterman et al., 1991). Thus it is possible that BP-1 secreted in response to dexamethasone could bind secreted IGF-I preventing its binding to insulin receptors. Alternatively, the binding of IGF-I by BP-1 may enable more efficient presentation of the ligand to the cell surface insulin receptor rather than sequestering it as suggested by some authors (Elgin et al., 1987). Adding further strength to my feedback loop hypothesis is the finding that insulin rapidly inhibits the expression of IGFBP-1 in H4IIE cells via its own receptor (Orlowski et al., 1991) and that the insulin effect is dominant over inducers (Unterman et al., 1991). Also des(1-3)IGF-I has recently been shown to inhibit production of BP-1 from a human hepatoma cell-line (Lindgren et al., 1993) Therefore, expression of

the IGFBP-1 gene is under similar control to the PEPCK gene in these cells. Hence, IGF-I and des(1-3)IGF could inhibit their own sequestration by inhibiting BP-1 production..

Although, I was unable to show expression of my constructs in the H4IIE cells it is necessary to realise that these cells, however well differentiated, do not behave as hepatocytes in culture. Thus, contrary to studies in H4IIE cells, in primary rat hepatocytes and fetal rat hepatocytes insulin is not able to exert a dominant inhibitory effect on PEPCK mRNA levels in the presence of PEPCK inducers such as cAMP, dexamethasone or glucagon (Christ et al., 1988; Iynedjian et al., 1989; Christ et al., 1990; Pegorgier et al., 1992). The reasons for this paradox in insulin action between hepatoma cells and normal hepatocytes are not known. Interestingly, work on cultured hepatocytes from mature rats supports my findings on inhibition of PEPCK expression by IGF-I. Hartmann et al., (1990) showed that IGF-I was able to antagonise the glucagon-dependent induction of PEPCK mRNA levels in primary rat hepatocytes. The antagonistic potency of IGF-I at half-maximal effective concentrations was 2 % of that exhibited by insulin and was probably mediated through the insulin receptor. Importantly, in this study the insulin and IGF-I effects were not dominant.

Obvious differences therefore exist between the regulation of the PEPCK gene in H4IIE cells and primary cultured hepatocytes. Furthermore, the situation *in vivo* differs from the *in vitro* state due to the presence of a circulatory system. While secretions from tissue culture cells remain in the near proximity of the cells, secreted products from an intact liver cell may be removed from the interstitial fluid into the bloodstream. These factors together suggest that the feeback loop operating to turn off expression from my construct in H4IIE cells would not be evident for PEPCK-IGF-I or PEPCK-des(1-3)IGF-I transgenes *in vivo*.

CHAPTER 4
PRODUCTION OF PEPCK-IGF-I TRANSGENIC ANIMALS

CHAPTER 4
PRODUCTION OF PEPCK-IGF-I TRANSGENIC ANIMALS

4.1 INTRODUCTION

Only a very small number of studies have looked at the expression of IGF-I in transgenic animals (Mathews et al., 1988; Behringer et al., 1990) and in these studies only transgenic **mice** have been produced. Mice are inappropriate as an endocrinological tool due to their small size. Nonetheless, IGF-I production in transgenic mice has been shown to have quite selective growth effects in these animals (Quaife et al., 1989). This size constraint may be overcome by using transgenic rats. The long term goal of transgenesis within our laboratory is to assess the ability of hIGF-I or IGF-I analogs to combat stress-associated weight loss in transgenic rats.

The levels of expression seen in transgenic mice using IGF-I cDNAs have been low relative to that achieved with other constructs (Mathews et al., 1988). This is not surprising when it is realised that the endogenous IGF-I genes are 50-80 kb in length (Rotwein et al., 1986; Shimatsu et al., 1987) and are likely to contain numerous regulatory regions within this enormous distance.

The PEPCK promoter (-574 bp- +73 bp) has been shown to be capable of directing tissue specific (liver/kidney) and dietary-inducible expression of the bGH gene in transgenic mice (McGrane et al., (1988)). Furthermore, in these transgenic mice, expression was inducible by glucocorticoid administration. As IGF-I levels have been shown to be negatively regulated by both undernutrition and stress (eg. glucocorticoids) (Phillips et al., 1984; Emler et al., 1987) it was suitable for us to use the Phosphoenolpyruvate Carboxykinase (PEPCK) promoter to direct IGF-I expression in our transgenic animals. Not only should we expect largely liver-specific expression of IGF-I but it would enable us to examine the effect of IGF-I produced in animals under stress. Additionally the PEPCK promoter should not be turned on until after birth (McGrane et al., 1988,1990) eliminating potential developmental problems due to inappropriate IGF-I expression *in utero*.

Having shown the expression of the PEPCK-IGF-Ia) construct in tissue culture cells, it was reasonable to expect its expression in transgenic animals. However, I had preliminary data suggesting that although our IGF-I cDNA expressed well in tissue culture cells (McKinnon et al., 1990), it expressed poorly *in vivo* even under the influence of a strong promoter (the RSV LTR). Thus, I had included in the PEPCK-IGF-Ia) construct

used in the following experiments, two heterologous introns from the rat PEPCK gene in an effort to maximize the expression of our cDNA *in vivo*. Several reports have shown some necessity for introns in attaining expression of cDNAs in transgenic mice (Brinster et al., 1988 ; Choi et al., 1991 ; Palmiter et al., 1991). As the feasibility of gene transfer to rats had now been established (Chapter 2), the PEPCK-IGF-Ia) construct was microinjected into both mice and rat oocytes and the resultant litters screened for the presence of the transgene.

This chapter describes the identification and characterisation of transgenic mice and rats carrying the PEPCK-IGF-Ia) transgene. All microinjection and embryo transfer work was carried out by Du Zhang Tao (Ph D. student in the Department of Obstetrics and Gynaecology, University of Adelaide). In addition, some of the acid gel filtration and subsequent radioimmunoassay described was carried out by Kirsty Moyse (Child Health Research Institute, Childrens Hospital, Adelaide).

4.2 RESULTS:

4.2.1 Generation and identification of transgenic animals

The PEPCK-IGF-Ia) construct was removed from its pBR322 based expression vector (see chapter 3, figure 3.2.4) by first linearizing with Sal I and then subsequent digestion with Sca I. Sal I cuts just 5' of the PEPCK promoter and Sca I cuts approximately 688 bps downstream of the hGH transcriptional termination signal. Thus the 2.96 kb linearized transgene contains approximately 700 bp of plasmid DNA. Due to the relatively large size of the transgene and the nature of its construction these two restriction sites were the only convenient options for transgene preparation. The transgene fragment was isolated and diluted to a concentration of 10ng/ml in PBS for microinjection. The same procedures as used for the MTIIa-pGH transgene were employed for generation of rats and mice with the PEPCK-IGF-Ia) construct (see materials and methods, 6.3.5).

From gene transfer into mice, an initial 37 pups were produced and later a further 18 animals generated. From transfer to rats 26 animals were generated. Small sections of tissue were taken from the tails of all animals at weaning and genomic DNA prepared as described in the Materials and Methods chapter (section 6.3.3).

Initial screening of the animals was by way of dot-blot analysis. Dot blots were carried out on 5 μg samples of genomic DNA from each animal. Blots were initially probed with a 487 bp Sma I/Dra I fragment from the hGH 3' region of the transgene (see figure

3.2.4). This probe contains largely 3'untranslated sequences and as such should be highly specific for the transgene. However, background hybridisation has been observed with this probe even at high stringency (65 °C in 0.1 x SSC/0.1% SDS), indicating its homology to sequences within the mouse and rat genome (probably the GH gene superfamily (Baumann et al., 1991)). Blots were subsequently stripped and re-probed with either the 615 bp Bam HI/Bgl II fragment of the PEPCK promoter or a Sal I/Bgl II fragment containing both the PEPCK promoter and IGF-I cDNA sequences. These probes were used only as a double check as they were also expected to hybridise to the endogenous PEPCK gene (especially in rats).

Figure 4.2.1 i) shows a dot blot carried out on the first 37 mice produced. This blot was probed with the Sma I/Dra I hGH 3' fragment and was washed very stringently before autoradiography. Animal 34 (row D column 11) clearly has several copies of the transgene (between 8-24 copies) while animals 29, 31, 32, 33, 35 , 36, 37 appear to have one or less copies of the transgene. The negative control DNA (row B column 1) on this blot showed no hybridisation to the probe. Reprobing of this filter with the Sal I/Bgl II fragment resulted as expected in a higher overall background, but again identified animal 34 clearly (data not shown).

Figure 4.2.1 ii) shows a dot blot of DNA from mice and rats probed with the hGH 3' probe. On this blot, mouse 34 (row B column 4) has been included as a positive control. In addition mouse 37 (row B column 5) was also included and showed much greater hybridisation to the probe on this occasion. Although the background hybridisation on this blot is quite high even after very stringent washing of the filter, it is obvious that a number of animals are exhibiting hybridisation above background. Rats, P1.4, P6.1, P6.3, P6.5 and P6.6 were above background, while mice 37, 39 and 40 were also clearly above background.

Reprobing of the filter in figure 4.2.1 ii) (Figure 4.2.1 iii)) with the PEPCKIGF-I, Sal I/Bgl II fragment gave interesting results. Although strong hybridisation was still observed in rat P1.4, P6.3 and P6.5, several animals previously identified with the hGH probe gave only background hybridisation with this probe for example P6.1, P6.6. Furthermore, this probe clearly identified animal P2.5 as transgenic. Similar differences were observed for the mice. This probe again showed mice 37, 39 and 40 to be transgenic but interestingly also identified animal 45 as having the transgene. Curiously, background hybridisation in mouse samples was much higher than in rat samples with this probe. These

results suggest that in some animals transgene sequences have been deleted during the integration process. Thus, while animals such as P6.3 appear to contain sequences from all parts of the transgene, others such as P2.5, lack sequences in the hGH region and others for example P6.1 may lack parts of the PEPCK promoter or IGF-I cDNA. Some of these results could also be explained by rearrangements of integrated transgenes. For example, rearrangement of the PEPCK promoter with respect to the IGF-I cDNA may lead to decreased hybridisation of this probe. Therefore, although the dot-blots identified a number of definite transgenics, I was unsure of any animals with low copy number integration of the transgene. To overcome this sensitivity problem I decided to use PCR to re-screen all animals.

4.2.2 Polymerase Chain Reaction analysis of transgenic animals

Oligonucleotide primers were designed to specifically amplify a region of the transgene between PEPCK promoter and the end of the IGF-I cDNA. Primers were analysed for their degree of complementarity, GC content and potential hybridisation to mouse and rat repetitive sequences using the PRIMER program in the GCG package of programs (6.3.24). The two primers chosen for use, encompassed 285 bps of transgene sequence and were deemed unlikely to hybridise with themselves or each other. Primer one was an 18 mer and mapped in the PEPCK promoter (materials and methods, 6.2.8) while primer two was a 20 mer and mapped in the leader peptide region of the IGF-I cDNA. Preliminary PCR was carried out using a number of different annealing temperatures and DNA from mouse 34, as a positive control along with plasmid DNA. Reaction conditions (see figure 4.2.2.i)) were finally chosen under which amplification of the 285 bp transgene fragment was only observed in transgenic animals or in reactions containing transgene DNA and no product was seen in reactions carrying DNA from control animals. The PCR was carried out on all 55 mice and 26 PEPCK-IGF-I rats produced.

In contrast to the low numbers of animals detected unequivocally in screening by dot-blot analysis, many more animals were detected using PCR. Following analysis 27/55 mice (49 %) and 11/26 rats (42 %) were found to possess the transgene including all of the animals previously shown by dot-blot as positive. Thus a very high percentage of animals in this study have incorporated the transgene. Although these figures are in excess of the percentages usually achieved for transgenesis (Kollias et al., 1992), it should be pointed out that screening of transgenics by PCR is only a relatively new technique and hence most

percentages presented in the literature may be an underestimate of the true number produced. The transgene-positive mice and rats are given below. These data have been summarised in Figure 4.2.2 i) which shows electrophoresis of PCR reactions carried out on mice and rats. Several rats are not present on this gel due to space restrictions but have been shown to be positive eg. (P2.7, P3.1, P6.3, P6.5, P6.6)

positive mice: 3, 4, 5, 6, 9, 10, 11, 12, 14, 18, 19, 21, 22, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 39, 45,

positive rats: P1.2, P1.4, P2.1, P2.2, P2.5, P2.6, P2.7, P3.1, P6.3, P6.5, P6.6.

As an additional assurance that the PCR product being made was indeed due to amplification of the transgene, a Southern transfer was carried out on the gel in figure 4.2.2 i) and the filter probed with a Bam HI/Bgl II fragment of the PEPCK promoter. Figure 4.2.2.ii) shows clearly that the major 285 bp product produced does indeed contain sequences from the PEPCK promoter. In a number of animals (see row 1 lanes d, e, i, j, row 2 lanes e, f, i and o) extra, smaller PCR products have been produced which hybridise to the PEPCK promoter probe. These may represent amplification of transgenes which have been deleted in regions between the two primer binding sites. Alternatively, these may be due to cross hybridisation of the primers with non-specific DNA leading to improper amplification. The higher molecular weight hybridisation seen in some tracks is likely to be due to hybridisation to the endogenous PEPCK gene carried over from the genomic template DNA used in the reactions.

4.2.3 Southern analysis of transgenic PEPCK-IGF-I mice

In order to examine the organisation of the integrated transgenes in the mice, Southern analysis was performed on DNA isolated from the tails of animals #34 and #37. These animals were chosen because of their higher copy number status. DNA was digested with enzymes that cut the transgene once or twice . Figure 4.2.3 shows the result of Southern analysis on 10 μg of genomic DNA. This Southern was probed with the Bam HI/Bgl II PEPCK promoter probe. Hence, both the endogenous PEPCK gene and the transgene are detected. Digestion of the negative control DNA with Bam HI gives rise to a 7 kb species only, due to hybridisation of the probe to the endogenous PEPCK gene (Yoo-Warren et al., 1983). In addition to this endogenous species, both animals 34 and 37 show a 2.9 kb species present. This band arises due to the head-to-tail integration of the PEPCK-IGF-I transgenes (greater than one copy) in the mouse genome. Bam HI has a unique site within

the transgene, 5' of the PEPCK promoter (see figure 3.2.4) and thus gives rise to a characteristic pattern of transgene length fragments when transgenes integrate 5'-3' next to one another. There are two Dra I sites within the transgene. The first site resides 522 bps from the start of the PEPCK promoter (or at position 1160 of the PEPCK gene), while the second site is present 487 bps into the hGH sequence (see figure 3.2.4). The negative control track reveals a band of approximately 2.6 kb, suggesting that there is a Dra I site 2 kb upstream of the endogenous PEPCK gene. This species is also observed as expected in animals 34 and 37. In both transgenic mice a smaller (faint) hybridising band of approximately 1.3 kb can be seen. A fragment of this size is predicted for head-to-tail integration of the PEPCK-IGF-I transgenes into the genome and represents hybridisation of the probe to a junction Dra I-Dra I fragment containing the 3' end of the transgene abutted to the 5' region of the adjoining transgene copy. The 1.7 kb internal Dra I fragment produced by digestion of each transgene copy would not be detected by the PEPCK promoter probe employed, since it lacks PEPCK promoter sequences. Hence in these two individuals the transgene appeared to have been integrated without rearrangement.

4.2.4 Growth of PEPCK-IGF-I mice

Mice were weighed from weaning onwards as it was possible that basal activity of the PEPCK promoter may elevate plasma IGF-I and lead to enhanced growth. McGrane et al., (1988) reported significant basal expression of bGH in their transgenic PEPCK-bGH mice, which led to enhanced growth. Such enhanced growth had also been previously observed in mice producing IGF-I from the MT-I promoter (Mathews et al., 1988a)).

When examining the mice during weighing there were no obvious physical differences between those animals with the transgene and those without. This is in contrast to the observation of increased head size in MT-IGF-I transgenic mice (Mathews et al., 1988a)). Although, significant variation was observed in the growth of individual mice, possession of the transgene did not confer any growth advantage overall to the transgenic group. In fact many animals with the transgene were considerably smaller than their non-transgenic littermates.

Figures 4.2.4 i) and ii) show the growth of transgenic males and females versus that of sex-matched non-transgenic littermates. Of all the transgenic mice produced animal # 37 (female) was the only animal to exhibit significantly increased growth after weaning. Figure 4.2.4 iii) shows that this animal continued to outgrow its other transgenic littermates

for some weeks after weaning. Therefore, this animal was the most likely basal expressor of IGF-I. Subsequent assay of plasma from this animal, however showed that it was not expressing (see section 4.2.5)

4.2.5 Detection of hIGF-I in transgenic animals

Mice and rats produce significant amounts of endogenous IGF-I subject to their nutritional intake and hormonal status (Emler et al., 1987; Goldstein et al., 1988; Luo et al., 1989; Thissen et al., 1991). Thus, before commencement of the transgenesis work it was necessary to have a screen which could distinguish transgene derived hIGF-I from endogenous mouse or rat IGF-I. The IGF-I family are highly conserved at the protein level, with only three amino acid differences between human and rat IGF-I/mouse IGF-I (Shimatsu et al., 1987; Murphy et al., 1987). Thus a polyclonal antibody assay would detect both endogenous IGF-I and transgene derived IGF-I together making assignment of expressing animals virtually impossible. A monoclonal antibody to hIGF-I was in use within our department (kindly provided by Dr. R. Baxter (Sydney University)). This antibody had previously been shown to cross react poorly with rat IGF-I (Dr. P.C. Owens personal communication; Tomas et al., 1992). Hence, it was reasonable to assume cross-reactivity would also be low with mouse IGF-I due to the latter's high sequence identity with rIGF-I (see introduction chapter, figure 1).

A second complication to measurement of transgene-derived hIGF-I in the mice/rats was the presence of IGFBPs in the circulation. If not removed prior to assay, IGFBPs are able to sequester the radiolabelled IGF-I tracer and/or bind to IGF-I in the test sample during the radioimmunoassay, leading to overestimates of IGF-I immunoreactivity. Thus dissociation and removal of IGFBPs is essential before plasma samples can be reliably assayed (Daughaday et al., 1987). Acidification of plasma and separation of free IGF-I by gel filtration chromatography under acidic conditions has been accepted and validated as the most reliable approach (Daughaday and Rotwein, 1989; Breier et al., 1990; Crawford, et al., 1992; Owens et al.,1990). Therefore, acid gel filtration chromatography was used in combination with the monoclonal antibody to set up the screen for hIGF-I (materials and methods, 6.3.20).

To validate this approach for measurements in mice an experiment was carried out in which three different samples were measured by radioimmunoassay (using the monoclonal antibody) following acidification and separation of IGFBPs from IGFs on a gel filtration

column. The three samples were 20 % pig plasma, 20 % mouse plasma and 20 % mouse plasma spiked with hIGF-I (4 μg/ml). Porcine IGF-I has an identical amino acid sequence to hIGF-I and therefore cross reacts with the monoclonal antibody. Samples were first defatted and then chromatographed before aliquots of each fraction were removed and assayed with the monoclonal antibody to hIGF-I.

Figure 4.2.5 i) shows the results of this experiment in diagramatic form. It can be observed that in all three samples there is a large peak of immunoreactivity eluting from the column at between 7-8 mls. This immunoreactivity is due to interference of the higher molecular weight IGFBPs in the assay (probably by way of tracer sequestration). The amount of IGFBP measured in each sample was very similar. Chromatography of iodinated recombinant hIGF-I under the same conditions on this column leads to a single peak of radioactivity eluting at between 9.5-10.0 mls. Hence a small amount of IGF-I immunoreactivity is being measured by the antibody in the 20 % pig plasma sample by virtue of the conservation of IGF-I sequence in this species. Importantly, no immunoreactive IGF-I was measured in the 20 % mouse plasma sample indicating that the monoclonal antibody could not recognise mouse IGF-I. Subsequent polyclonal antibody assay confirmed the presence of immunoreactive mIGF-I (150-300 ng/ml) in these fractions. When mouse plasma was spiked with hIGF-I (4 μg/ml) the antibody again detected IGF-I in fractions between 9.5-10 ml, confirming that this assay could distinguish hIGF-I from mIGF-I and validating this approach for use on mice/rat plasma samples.

Mice were starved overnight before blood sampling in an effort to maximize any expression of IGF-I from the PEPCK promoter (Hopgood and Ballard, 1973; Yoo-Warren et al., 1981; Girard et al., 1991). Due to the size of the mice only a small volume of blood was able to be sampled. Plasma was diluted to 10 % and defatted prior to chromatography. Chromatography of radioactive IGF-I was performed before and after sample chromatography to verify the elution position of IGF-I. Four pools of eluent were collected and aliquots of these pools assayed with the monoclonal antibody. Unfortunately, essentially no hIGF-I was found in any of the 55 mouse samples assayed. Hence it was likely that none of the animals with the transgene were expressing hIGF-I. However, it was also possible that the starvation period of 24 hrs was insufficent to induce IGFBPs in these animals. In such circumstances any hIGF-I produced may have been cleared quickly from the body. Alternatively the levels of hIGF-I being produced were below the level of detection of the assay used (less than 70 ng/ml).

4.2.6 Analysis of PEPCK-IGF-I mice for hIGF-I mRNA

Since no evidence was found for hIGF-I in the plasma of mice shown to possess the PEPCK-IGF-Ia) transgene it was decided to sacrifice a number of animals and examine their tissues for evidence of transgene expression. Animals were again fasted for 24 hrs prior to sacrifice to induce any PEPCK promoter directed hIGF-I expression. RNA was extracted from the livers and kidneys of all animals and the adipose tissue of 10 animals. In total 12 transgenic and 4 non-transgenic mice were assayed for the presence of hIGF-I mRNA. The positive control used in each assay was total RNA isolated from PEPCK-IGF-Ia) or RSV-IGF-I expressing Chinese Hamster Ovary cell lines described in chapter 3. The 159 bp riboprobe used has been previously described and contains the E domain of the IGF-I cDNA, a region which diverges between species (Jansen et al., 1983; Bell et al., 1986; Rotwein et al.,1986; Shimatsu et al., 1987). Despite the protection of the predicted 102 bp species in the positive control used, no protected species was observed in RNA from any of the mice assayed (see figure 4.2.9 i)). Therefore, in line with results of the RIA none of the PEPCK-IGF-Ia) mice were expressing hIGF-I. As not all transgene positive mice were assayed for IGF-I mRNA it is possible that some of the unassayed animals were low level expressors of the transgene.

4.2.7 Analysis of PEPCK-IGF-Ia) rats

As discussed in section 4.2.1, 26 rats were produced by microinjection of the PEPCK-IGF-Ia) construct. By PCR analysis 11/26 were shown to have incorporated the transgene. In view of results obtained in screening mice for expression of hIGF-I it was decided to try and induce transgene expression to greater levels before sampling and assaying the plasma of each rat. Thus, a protein-rich /carbohydrate poor diet was produced and fed to the rats ad libitum for a 5 day period prior to blood sampling. The diet was similar to that used by McGrane et al., (1988) to induce expression of their PEPCK-bGH transgene. It contained 64 % casein, 22 % methyl cellulose, 11 % vegetable oil, 2 % yeast and a 1 % mineral mix with vitamins. Rats had free access to water throughout the 5 day period.

Weighing of the animals prior to blood sampling revealed that in all cases weight loss had occurred (between 5-10 g) (data not shown). Hence it is likely that many of the animals did not consume the diet at all. Blood was sampled before commencement of the diet and

immediately after removal of the diet. As with samples from the PEPCK-IGF-Ia) mice, each plasma sample was chromatographed by gel filtration under acidic conditions to separate any IGFBPs, before being assayed with the hIGF-I monoclonal antibody. No hIGF-I was detected in the plasma of any of the rats assayed by this means. The minimal detectable concentration in this assay corresponded to approximately 50 ng/ml hIGF-I. Because of the lower sensitivity of the monoclonal antibody assay, all fractions were reassayed using a more sensitive polyclonal antibody to hIGF-I (Tomas et al., 1991a). This antibody cross reacts highly with endogenous rat IGF-I, but has a lower level of detection and suffers less from interference due to IGFBPs. Table 4.2.7 shows the levels of immunoreactive IGF-I detected in the rat samples with the polyclonal antibody. Thus, unlike results with the monoclonal antibody, significant levels of immunoreactive IGF-I were detected in the polyclonal assay, all of which should be due to endogenous rat IGF-I. A minimal detectable concentration of 5 ng/ml confirmed that this assay was more sensitive than the monoclonal antibody assay.

Interestingly, out of 26 animals all but two of the animals exhibited a decrease in plasma IGF-I following the diet. Malnutrition in rats is generally associated with a decrease in serum IGF-I levels, as is fasting (Emler et al., 1987; Straus et al., 1988; O'Sullivan et al., 1989). It is also known that protein restriction in rats reduces serum IGF-I levels (Maiter et al., 1988; Tomas et al., 1990; Thissen et al., 1990). However, no reports have been made on the effect of low carbohydrate/high protein diets on rat IGF-I levels. The observed decrease in plasma IGF-I in this group of rats supports the suggestion that many of the animals may not have eaten the diet. Therefore, it was highly unusual that two animals responded to the diet with increased plasma IGF-I. It should be noted that both of these animals exhibited concomitant weight loss during the 5 day period and therefore would be expected to have lowered rat IGF-I levels. Furthermore, both animals P2.5 and P6.3 had been previously shown to have integrated the transgene. Hence, the increased IGF levels observed in these animals may have been due to the expression of hIGF-I from the transgene in response to the lowered carbohydrate diet. However, my calculations suggested that were the increased polyclonal immunoreactivity in P2.5 and P6.3 due solely to hIGF-I, these animals should have been detected in the initial monoclonal assay. Despite these calculations it was possible that the monoclonal antibody assay was not sensitive enough to detect this amount of hIGF-I. Therefore, both animals were bred as potential transgenic expressors before more detailed analysis of their blood IGF-I was made.

4.2.8 Inheritance of the PEPCK-IGF-Ia) transgene in rats

Breeding of both P2.5 and P6.3 led to F1 generations. P2.5 gave birth to a litter of 13 animals (8 males and 5 females) while P 6.3 sired a litter of 12 (9 males and 4 females) animals. PCR was used to screen for the presence of the transgene in the F1 animals. Although 6/12 animals from the F1 of P6.3 were found to have inherited the transgene, none of the offspring of P2.5 were identified as having the transgene. Figure 4.2.8 i) shows PCR identification of the carriers of the transgene amongst the F1 offspring of P6.3. The transgene was amplified by the primers in animals 6.3.2, 6.3.5, 6.3.6, 6.3.7, 6.3.10 and 6.3.13 (not visible on photo).

To ensure that the organisation of the transgene had been maintained in the F1 animals, Southern analysis was carried out (figure 4.2.8). Bam HI digests the transgene at a single site 5' of the PEPCK promoter and thus should give rise to transgene length, 2.96 kb fragments if head-to-tail integration of more than one transgene has occurred and 5.8 kb fragments if tail-to-tail integration has occurred. Figure 4.2.8 ii) shows Bam HI Southern analysis of the P6.3, F1 generation. This filter was probed with the Bam HI/Bgl II PEPCK promoter probe which cross-hybridises to the 7 kb Bam HI fragment of the endogenous rat PEPCK gene, shown in the negative control lane. This species is also visible in many of the other tracks. The autoradiograph shows P6.3 to have both 2.96 kb and 5.8 kb hybridising species, indicative of both head-to-tail and tail-to-tail integrations of the transgene. Although it appears here that only the lower molecular weight fragment has been passed on to animals in the F1 generation, later Southern analysis (see figure 4.2.11i)) showed that both the 2.96 and 5.8 kb species were indeed inherited. While P6.3.5, P6.3.6, P6.3.7 and P6.3.10.have clearly inherited the transgene, longer exposure of the film was required to detect the 2.9 kb band in animals P6.3.2 and P6.3.13.

4.2.9 Testing expression of PEPCK-IGF-Ia) in the P6.3 family

Having bred the potentially expressing PEPCK-IGF-Ia) rats, it was decided to sacrifice the founder animals for analysis of their RNA. Thus, after fasting overnight, rats P1.1(non-transgenic), P2.2, P2.5 and P6.3 were sacrificed and their livers, kidneys and intestines taken for RNA extraction. In addition, a large volume (1-1.5 mls) of blood was

taken from each animal by heart puncture immediately prior to death, and plasma prepared for IGF-I assay.

Following RNA extraction, samples from all four animals were included in a Ribonuclease protection assay which utilized the hIGF-I 3' riboprobe described earlier (section 3.2.4). In these assays RNA from a hIGF-I expressing Chinese Hamster Ovary cell-line was used as a positive control. Initially only liver RNA was assayed for hIGF-I.

Figure 4.2.9 i) shows an assay in which RNA from the livers of ten PEPCK-IGF-Ia) mice were also included. Only liver RNA from rat P6.3 gives rise to the correctly protected 102 bp species (lane 16). Hence, P6.3 was transcribing the PEPCK-IGF-I transgene following fasting. To further examine the transgene's expression pattern, an assay was carried out on RNA from the kidneys and intestines of animals P2.5 and P6.3. Figure 4.2.9 ii) shows that RNA from all three tissues in rat P6.3 protect the 102 bp species. Since equal amounts of RNA were assayed in each tissue it is clear that expression of the transgene was highest in the liver (lane 6). Expression was somewhat lower in the kidney (lane 5) and lower still in the intestine (lane 7). This pattern of expression is similar to that observed for bGH transcription in PEPCK-bGH transgenic mice (McGrane et al., 1988) and corresponds to the expression preference of endogenous PEPCK (McGrane et al., 1990; Short et al., 1992; Watford et al., 1988).

Plasma taken at sacrifice from P1.1, P2.5 and P6.3 was defatted and chromatographed by gel filtration under acid conditions to separate IGFBPs from IGF. Due to the limited availability of blood taken from the tail vein initially, injection of only 10 % plasma had been permitted, possibly limiting our detection of transgene derived hIGF-I. Therefore, due to the larger volume of blood taken, 50 % plasma was injected onto the column. Fractions were collected and triplicate aliquots of each fraction assayed with the hIGF-I monoclonal antibody. Figure 4.2.9 iii) shows the assay results across the chromatography profile. A large amount of interference in the assay is seen due to the IGFBPs eluting between 7-9 mls. Importantly fractions between 9-10 mls were found to contain immunoreactive hIGF-I in the P6.3 sample (approximately 32 ng/ml). This value was just above the minimal detectable concentration for this particular assay of 27 ng/ml No such immunoreactivty was observed in samples from animals P1.1 and P2.5. Hence, this assay confirmed that P6.3 was a transgenic expressor.

Having already produced an F1 generation from this founder animal, half of which had inherited the transgene it was reasonable to expect expression of hIGF-I in all of these

animals. With this in mind the entire F1 generation of P6.3 were fasted for 24 hours in an effort to induce hIGF-I expression from the PEPCK promoter. Large volume blood samples were taken after the fast and 24 hours following refeeding, via the tail vein of the animals. As previously, 50 % plasma was defatted and chromatographed on a gel filtration column. Four pools of eluant were collected from each sample and aliquots assayed for hIGF-I with the monoclonal antibody. Radiolabelled hIGF-I standard was chromatographed before and after the samples to verify the elution position of the IGF-I species. The results of this assay showed that with the exception of three animals, the rats showed no immunoreactive hIGF-I in their blood before or after fasting. The three animals in question, P6.3.5, P6.3.6 and P6.3.7 had all been shown to have the transgene (see figure 4.2.8 ii)). After fasting, the levels of hIGF-I were 11.8 ng/ml, 11.2 ng/ml and 34 ng/ml respectively. Following refeeding the levels in P6.3.5 and P6.3.7 decreased to 0.3 ng/ml and 9.6 ng/ml while P6.3.6 showed no real change in its level (13.4 ng/ml compared to 11.2 ng/ml). All other plasma samples in this assay were below the lowest hIGF-I standard used (16pg/tube). Thus, the absolute levels of blood-borne hIGF-I in the F1 offspring were somewhat variable despite the inheritance of the same transgene from P6.3 in each case. This phenomena has been previously observed in transgenic animals (Palmiter et al., 1982; McGrane et al., 1988) The detection of hIGF-I following fasting and decreased expression of the transgene following re-feeding in P6.3.5 and P6.3.7 indicates expression of hIGF-I in these animals parallels the expression pattern observed for rat PEPCK (Hopgood et al., 1973; Yoo-Warren et al., 1981) and suggests hIGF-I is under the control of the PEPCK promoter. The relatively constant level of hIGF-I detected in P6.3.6 after fasting and following re-feeding is however not consistent with this suggestion. In order to establish the mode of transgene control in these animals further analysis of the F1 animals will need to be carried out.

To establish that the increase in plasma hIGF-I following fasting in P6.3.5 was due to transcription of the transgene, this animal was fasted overnight and sacrificed the following day. An F1 animal known not to possess the transgene, P6.3.8 was also fasted and sacrificed as a control. Total RNA was extracted from the liver, kidney, intestine and hearts of both animals before Ribonuclease protection assays were carried out using the 159 bp hIGF-I specific probe. Figure 4.2.9 iv) shows that P6.3.5 was indeed expressing hIGF-I mRNA following the fast, in both the liver (lane 5) and the kidney (lane 6). Only low level expression was observed in the intestine of this animal (lane 7). Importantly, no expression of hIGF-I mRNA was observed in the heart of this animal suggesting that

expression of hIGF-I was restricted to those tissues known to express PEPCK. RNA from founder P6.3 included in this protection assay, exhibited are very similar level of hIGF-I mRNA in both liver (lane 2) and kidney (lane 3) to P6.3.5. Samples from non-transgenic rat P6.3.8 showed no protected species in this assay as expected.

4.2.10 IGFBPs in PEPCK-IGF-Ia) rats

Since alteration in the production of IGFBPs has been shown in both GH and IGF-I expressing transgenic mice (Camacho-Hubner et al., 1991) it was of interest to examine this aspect in the PEPCK-IGF-Ia) transgenic rats. Therefore blood samples taken after fasting and following re-feeding of the F1 generation PEPCK-IGF-Ia) rats were analysed for their content of IGFBPs by Western ligand blotting (see section 6.2.23). Figure 4.2.10 shows a ligand blot probed with radiolabelled IGF-2 tracer. In all tracks a triplet (40-50 kDa) and a doublet (28-30 kDa) of binding proteins was observed. The lower molecular weight 24 kDa often observed in rat plasma samples (see Chapter 2, 2.2.8) was virtually undetectable . In each animal examined the total amount of IGFBP (all species) was higher after fasting than following re-feeding. In some cases, (P6.3.5 and P6.3.8) IGFBP levels were greatly decreased during re-feeding. There was not any observable difference in the behaviour of the IGFBPs in plasma from transgenic as opposed to non-transgenic animals. To examine in more detail the IGFBP changes observed in animals during expression of hIGF-I, densitometry was carried out on this autoradiograph. The levels of total IGFBP in each fasted animal were normalised to the total levels after re-feeding. In the control animal (P6.3.8), total IGFBP levels increased by 98 % after fasting. In P6.3.5, P6.3.6 and P6.3.7 IGFBP increased by 179 %, 36 % and 6 % respectively. Therefore there was no direct relationship between the expression of hIGF-I and IGFBP changes in the fasted state. It is noteworthy that P6.3.7 which exhibited the highest expression of hIGF-I during fasting also showed the smallest induction of IGFBPs in this state. This was due primarily to a higher level of IGFBP in the refed state. The higher levels of IGFBP in both P6.3.6 and P6.3.7 during re-feeding is consistent with these two animals expressing higher levels of hIGF-I in this state than P6.3.5. However such alterations in the total level of binding protein may be related more to changes in the level of rat IGF-I than the transgene IGF-I in these states.

4.2.11 Breeding of PEPCK-IGF-Ia) transgenic rats

Successful breeding of P6.3 5, P6.3.6 and P6.3.7 yielded three litters of 12, 13 and 10 animals respectively. Genomic DNA was made from tail samples taken at weaning and PCR (see section 6.3.3) used to initially screen the animals for the transgene. Figure 4.2.11i) shows PCR analysis of the F2 generation of P6.3.5 in which 8/13 (62 %) animals were found to have inherited the transgene. PCR analysis on the P6.3.6 litter suggested 12/13 (92 %) had inherited the transgene from their parent while in the case of P6.3.7, 5/13 (38 %) had inherited the gene (data not shown).

To confirm inheritance of the transgene in these animals and to ensure that the same transgene architecture had been maintained in passage through the germline, Southern analysis was performed on the F2 generation animals. Bam HI was used to digest DNA and the filters were probed with the Bam HI/Bgl II PEPCK promoter fragment.

Figure 4.2.11 ii) shows Bam HI Southern analysis of animals in the F2 generations of P6.3.5 and P6.3.7. Two distinct species are detected with the PEPCK promoter probe. The lower, 2.9 kb species represents head-to-tail integration of the transgenes while the higher molecular weight species of approximately 5.8 kb is due to tail-to-tail transgene integration. In some lanes a 7 kb species corresponding to the endogenous PEPCK gene can be seen. Comparison of the pattern exhibited by the F1 animals with their F2 offspring shows that the organisation of the transgene has been stably inherited through the F2 generation. This Southern blot confirmed that 5/13 animals produced from P6.3.7 had inherited the transgene. Further Southern analysis on the F2 generation of P6.3.6, shown in figure 4.2.11 iii) confirmed that 12/13 of these animals had inherited the transgene structure of their parent. Hence the PEPCK-IGF-Ia) transgene has been stably inherited throughout two generations of animals.

To examine expression of the transgene in the F2 generation animals, three individual males (6.3.5.4, 6.3.5.5 and 6.3.5.6) from one family were fasted for 24 hours and sacrificed the following day. RNA was extracted from liver, kidney, intestine and heart of these animals before expression was evaluated in a ribonuclease protection assay using the previously discussed hIGF-I 3' riboprobe. Figure 4.2.9 v) shows that both males 6.3.5.5 and 6.3.5.6 which had inherited the transgene expressed the hIGF-I mRNA species predominantly in the liver and at slightly lower levels in the kidney. No expression of hIGF-I was observed in the non-transgenic animal, 6.3.5.4, nor was any expression observed in the hearts and intestines of the transgenic rats. This result has been verified in

three independent assays. The faint bands observed in tracks 1-4 and 12 were also present in a protection containing only yeast RNA and the probe. These species are believed to be due to probe secondary structure and are only observed after long periods of autoradiography.

4.2.12 Growth of PEPCK-IGF-Ia) transgenic rats.

Since expression of hIGF-I in transgenic mice had been shown to result in increased growth of these animals (Mathews et al., 1988) it was possible that such increased growth could be manifested in the PEPCK-IGF-Ia) rats. Thus, the F1 generation animals produced from mating of P6.3, were weighed from weaning as were F2 animals produced from mating of P6.3.5, P6.3.6 and P6.3.7. These weekly weights were expressed as a percentage of the animals original body weight at weaning.

Figure 4.2.12 i) illustrates the growth of primary PEPCK-IGF-Ia) rats. When the growth of the hIGF-I expressing rat , P6.3 was compared to the growth of two sex-matched non-transgenic littermates (P6.1 and P6.2), no significant difference was found. Rather than exhibiting enhanced growth, this animal shows a small inhibition of growth compared to the controls. Thus the presence of the PEPCK-IGF-Ia) transgene was not positively influencing growth. It should be remembered that hIGF-I had only been detected in the plasma of P6.3, following fasting. Therefore it is possible that in the fed animal hIGF-I may be virtually absent due to its control by the insulin-repressible PEPCK promoter (Sasaki et al., 1984; Magnuson et al., 1987; Forest et al , 1990). It should also be noted that the levels of hIGF-I detected in the this rat were relatively low (34 ng/ml) and as such may have been ineffective in eliciting a biological response. Alternatively, an inhibition of growth could be achieved due to repression of endogenous rat IGF-I transcription by hIGF-I. Such feedback inhibition of IGF-I trascription has been observed following IGF-I infusion into rats (Schalch et al., 1989) and due to hIGF-I expression in transgenic mice (Mathews et al., 1988).

Figure 4.2.12 ii) shows the growth performance of the F1 generation offspring of P6.3, compared to littermates that have inherited the transgene but were not found to be expressing hIGF-I As with P6.3, no significant differences were observed between those animals shown to be expressing hIGF-I and those which lacked expression. Although P6.3.6 is growing at a faster rate than most other animals, consistent with its expression of hIGF-I, P6.3.13 which was not found to be expressing also grew at a similar rate. Thus, it

is unlikely that this moderate enhancement of growth was due to transgene derived hIGF-I. In support of this it can be seen that rats P6.3.5 and P6.3.7 were slower growers than animals lacking expression of hIGF-I. Thus, the growth rates of all animals which inherited the transgene were not significantly different from animals lacking the transgene. These results suggest that the level of hIGF-I produced in this family of transgenic rats during normal dietary intake is ineffective in promoting total body growth.

FIGURE 4.2.1 i) Samples (5 µg) of genomic DNA from PEPCK-IGF-Ia) mice 1-37 were denatured and applied to Nytran™ filters. A series of copy number controls was included containing PEPCK-IGF-Ia) plasmid diluted in 5 µg of non-transgenic mouse DNA (NT) to give the equivalence of 1-80 copies of the transgene/cell assuming a rat/mouse genome size of 1x 10^6 bps. A value of 12.5 pg of plasmid DNA was determined to be equivalent to 1 transgene copy /haploid genome in 5 µg DNA. The values in brackets refer to pg of plasmid DNA. This blot was hybridised with the oligolabelled Sma I/Dra I 3'fragment of the hGH gene and was washed at 65°C in 0.1 x SSC/ 0.1 % SDS for 30 minutes before overnight autoradiography. A key to sample identity is provided below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1(12.5)	2(25)	4(50)	8(100)	24(300)	32(400)	40(500)	80(1000)				
B	NT	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
C	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22	#23
D	#24	#25	#26	#27	#28	#29	#30	#31	#32	#33	#34	#35
E	#36	#37										

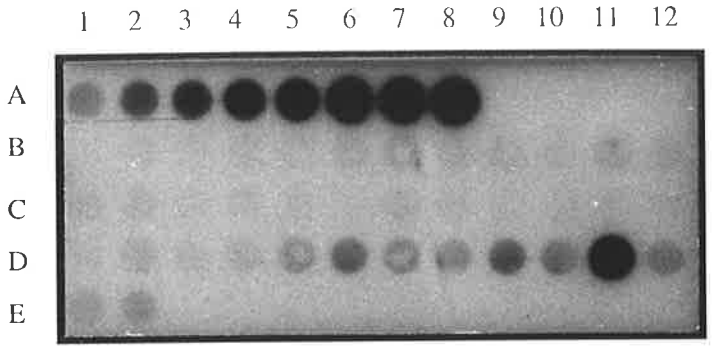
FIGURE 4.2.1 ii) Dot blot was performed on 5 µg of genomic DNA from PEPCK-IGF-Ia) rats (P1.1-P6.6) and mice #38-#55. This blot was carried out as for figure 4.2.1 i) and was probed with the Sma I/Dra I hGH 3' fragment. The filter was washed at 65°C for 1 hr. in 0.1 x SSC/ 0.1 % SDS before overnight autoradiography. The samples applied were as below

	1	2	3	4	5	6	7	8	9	10	11	12
A	1(12.5)	2(25)	4(50)	8(100)	24(300)	32(400)	80(1000)					
B	NT(rat)	NT(mouse)	NT(rat)	#34	#37							
C	P1.1	P1.2	P1.3	P1.4	P2.1	P2.2	P2.3	P2.4	P2.5	P2.6	P2.7	P3.1
D	P3.2	P4.1	P4.2	P4.3	P4.4	P4.5	P4.6	P4.7	P6.1	P6.2	P6.3	P6.4
E	P6.5	P6.6	#38	#39	#40	#41	#42	#43	#44	#45	#46	#47
F	#48	#49	#50	#51	#52	#53	#54	#55				

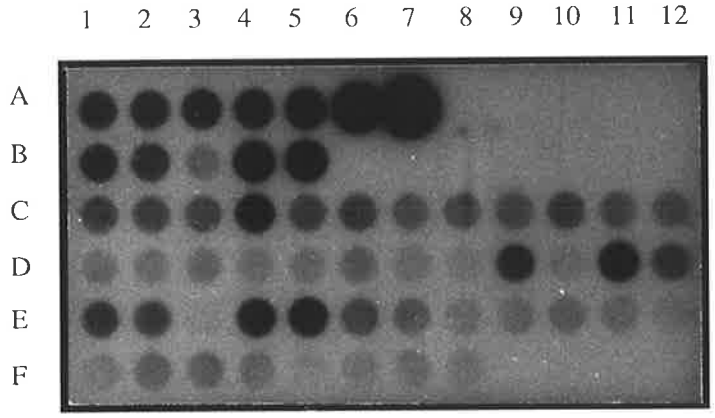
FIGURE 4.2.1 iii) The filter in 4.2.1 ii) was stripped and re-probed with the Sal I/ Bgl II fragment of the PEPCK-IGF-Ia) construct. The filter was washed as above and was autoradiographed overnight.

Dot-Blot Analysis of PEPCK-IGF-1 Transgenic Animals

i)



ii)



iii)

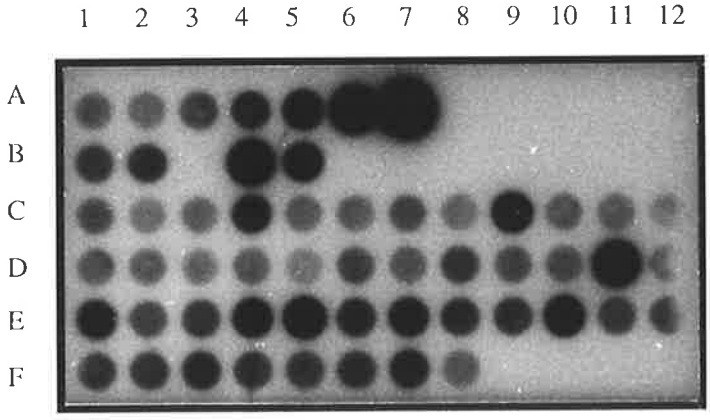


FIGURE 4.2.2 i) Polymerase Chain Reaction was carried out on PEPCK-IGF-Ia) mice and rats to confirm which animals were transgenic. The primers used for amplification were derived from both the PEPCK promoter and the IGF-I cDNA. PCR was carried out in a total volume of 50 µls (see materials and methods section 6.3.13). Reactions were overlayed with paraffin oil before the following cycles were carried out in a Cetus-Emler Thermo cycler.

1 cycle of denaturing at 94°C for 4 minutes.

29 cycles at 94°C for 45 seconds / annealing at 58°C for 1 minute / extension at 72°C for 1 minute

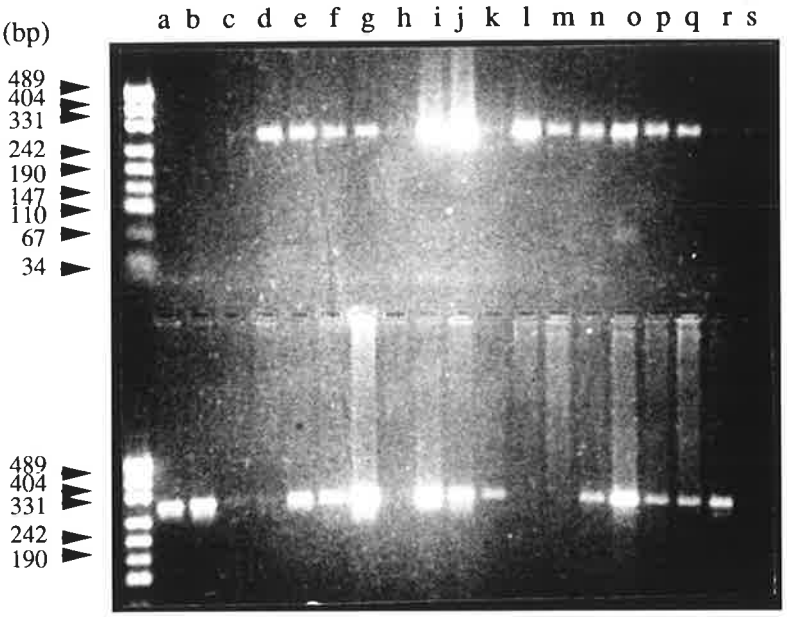
Following these cycles, 5 µl aliquots of each reaction were electrophoresed on 2 % Agarose TBE buffered gels and the reaction product visualised by UV irradiation of the Ethidium Bromide stained gels. Each set of PCR reactions contained contamination controls lacking addition of template DNA. Also included were negative controls containing non-transgenic mouse or rat genomic DNA and a positive control containing the microinjected transgene DNA. The PCR product was sized by comparison with co-electrophoresed Hpa II cut pUC DNA markers (low molecular weight range).

FIGURE 4.2.2 ii) The 2 % gel shown in 4.2.2 i) was transferred overnight to Nytran™ membrane and the DNA cross-linked to the filter. The filter was subsequently probed with the oligolabelled Bam HI/ Bgl II fragment of the PEPCK promoter and washed at high stringency (0.1 x SSC/0.1 % SDS at 65°C for 30 minutes) before autoradiography overnight. The loadings for each lane were as below: (NTm=non-transgenic mouse ; NTr =non-transgenic rat; transg.=PEPCK-IGF-Ia) Sal I/Sca I transgene DNA)

	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s
1.	no DNA	NTm	#3	#4	#5	#6	#9	#10	#11	#12	#14	#18	#19	#21	#22	#25	#26	#28	transg.
2.	#29	#30	#31	#32	#33	#34	#35	#36	#37	#39	#45	NTr	P1.1.	P1.2	P1.4	P2.1	P2.2	P2.5	P2.6

PCR Identification of PEPCK-IGF-I Transgenic Animals

i)



ii)

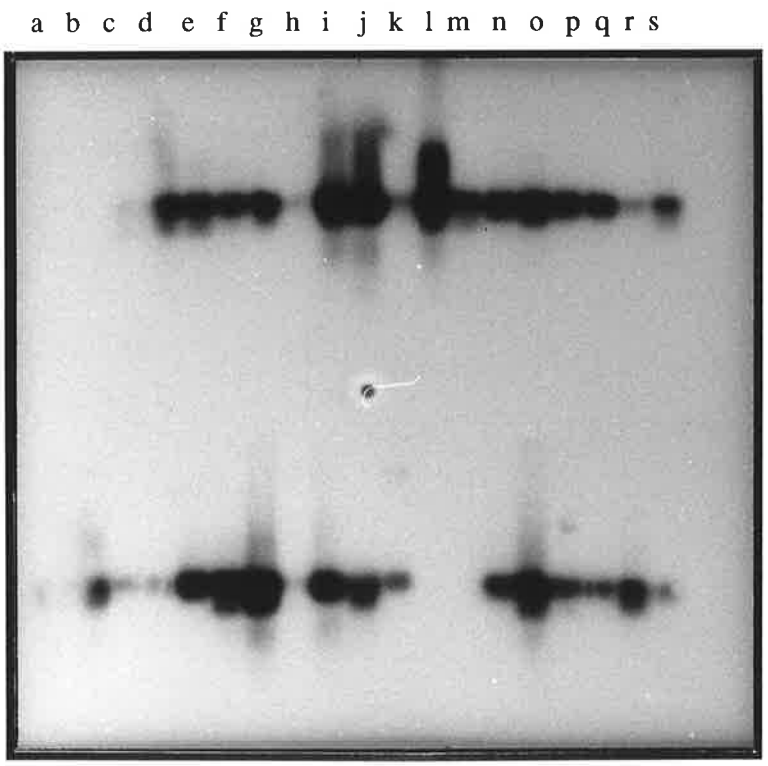


FIGURE 4.2.3 Southern analysis was carried out on DNA from transgenic PEPCK-IGF-Ia) mice #34 and #37. Genomic DNA (20 μg) was digested with either Bam HI or Dra I and electrophoresed on 0.8 % TBE Agarose gels. The gel was then transferred to Nytran™ membranes and DNA crosslinked by UV irradiation to the filter. This Southern was probed with the oligolabelled Bam HI/ Bgl II PEPCK promoter fragment and the filter was washed at 42°C in 0.1 x SSC/ 0.1 % SDS for 30 minutes before autoradiography for 72 hours. The sizes of radiolabelled SPP1 markers co-electrophoresed on the gel are given on the left hand side. The lanes are as described below.

lane 1: non-transgenic mouse (Bam HI)

lane 2: #34 (Bam HI)

lane 3: #37 (Bam HI)

lane 4: non-transgenic mouse (Dra I)

lane 5: #34 (Dra I)

lane 6 #37 (Dra I)

Southern Analysis of PEPCK-IGF-1a) Mice

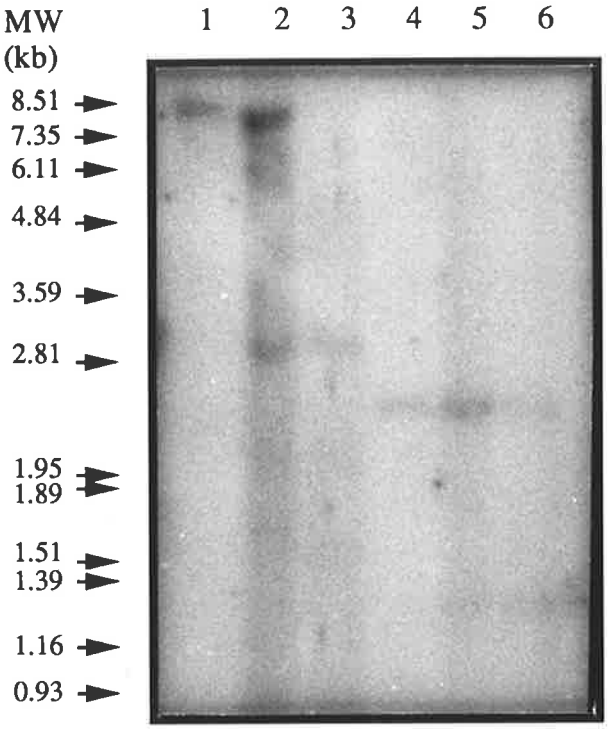
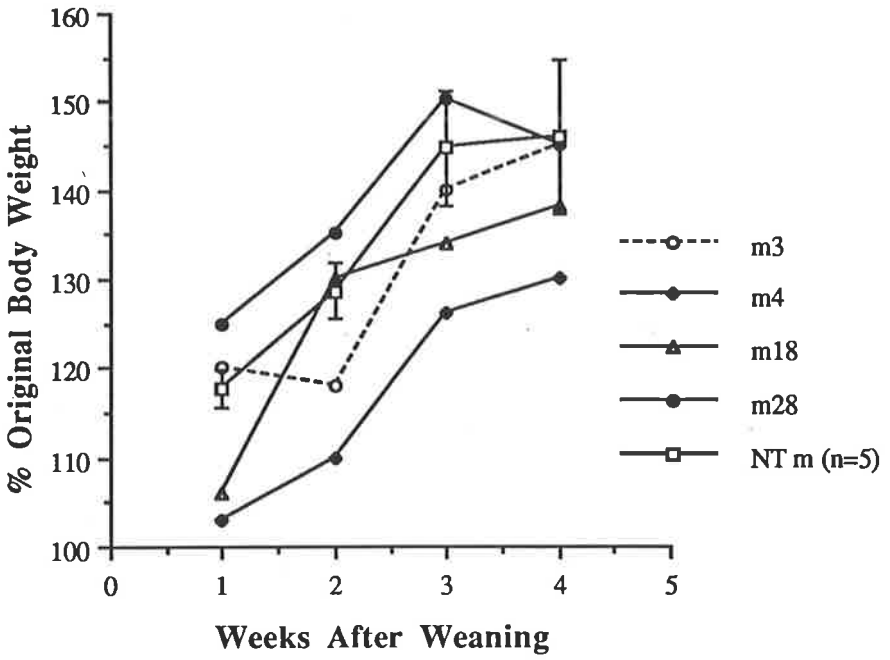


FIGURE 4.2.4 i) Growth curves from weaning of several transgene positive male PEPCK-IGF-Ia) mice compared with non-transgenic littermates. Body weights were expressed relative to the weight of each animal at weaning.

FIGURE 4.2.4 ii) Growth curves from weaning of several female PEPCK-IGF-Ia) transgenic mice compared to their non-transgenic littermates. Weights were expressed relative to the body weight of each animal at weaning.

Growth of Male PEPCK-IGF-1a) Mice



Growth of Female PEPCK-IGF-1a) Mice

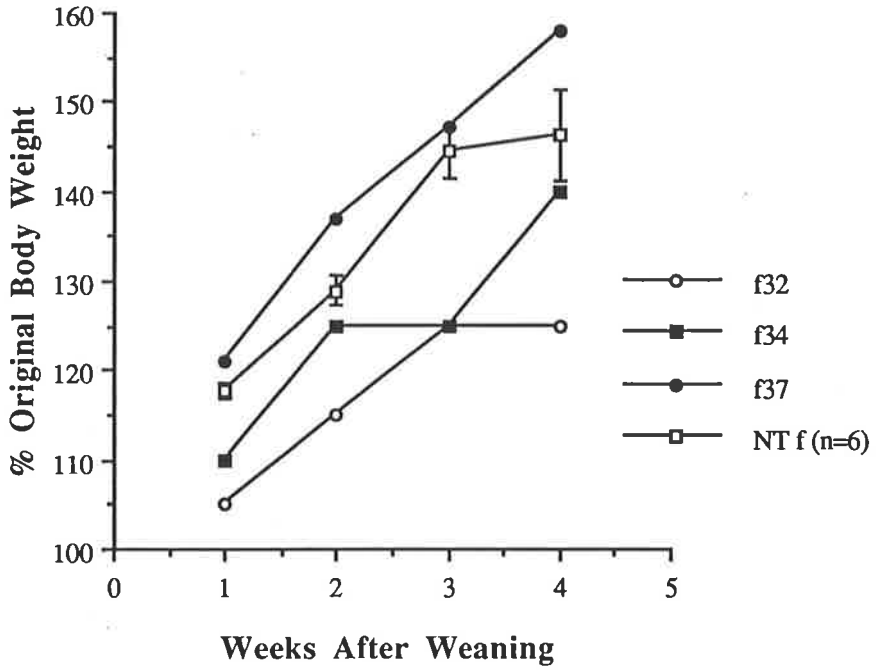


FIGURE 4.2.5 i) Two hundred microlitres of 20 % plasma was chromatographed by gel filtration under acidic conditions to separate IGFBPs from IGF-I. Fractions (200µl) were collected throughout the run and 50 µl aliquots of each fraction assayed in triplicate for hIGF-I using a monoclonal antibody radioimmunoassay (Tomas et al., 1992). Three separate chromatography runs were carried out on 20 % mouse plasma, 20 % porcine plasma and 20 % mouse plasma spiked with hIGF-I (4 µg/ml). The elution position of hIGF-I (9-10 ml) was determined by chromatography of radiolabelled hIGF-I standard before and subsequent to the plasma samples. The immunoreactivity eluting at 7-8ml corresponds to IGFBP interference in the radioimmunoassay.

Acid Gel Filtration of Plasma

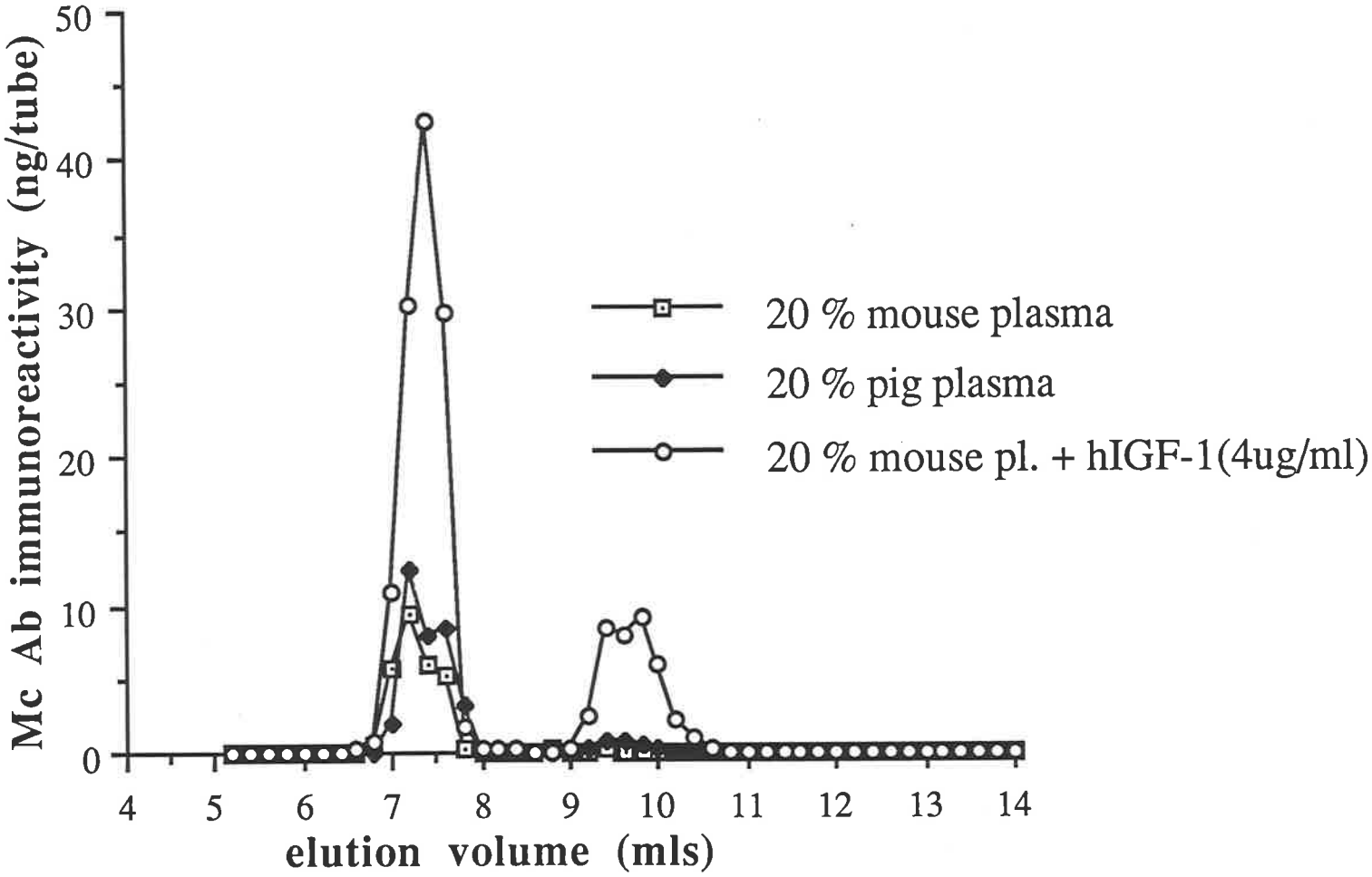


FIGURE 4.2.7 All PEPCK-IGF-Ia) rats were placed on a high protein/low carbohydrate diet containing 64 % casein, 22 % methyl cellulose, 11 % vegetable oil, 2 % yeast and a 1 % mineral mix +vitamins, for a period of 5 days. Blood samples were taken prior to and directly after this period and were assayed using a polyclonal IGF-I radioimmunoassay. n.d.=not determined. These results represent means of triplicate determinations on each sample.

Rat I.D.	IGF-I immunoreactivity (ng/ml) Before Diet	After Diet
P1.1	538	25
P1.2	548	504
P1.3	472	440
P1.4	474	371
P2.1	776	429
P2.2	1144	660
P2.3	739	603
P2.4	657	372
P2.5	**477**	**646**
P2.6	455	413
P2.7	333	197
P3.1	892	653
P3.2	271	n.d
P4.1	662	580
P4.2	808	676
P4.3	760	703
P4.4	756	521
P4.5	690	598
P4.6	553	468
P4.7	465	375
P6.1	858	707
P6.2	804	787
P6.3	**275**	**863**
P6.4	854	n.d
P6.5	735	518
P6.6	586	472

FIGURE 4.2.8 i) Polymerase Chain Reaction was carried out to identify transgenics amongst the F1 progeny of PEPCK-IGF-Ia) rat,P6.3. The two primers employed, mapped to the PEPCK promoter and IGF-I cDNA respectively and amplified up a 285 bp region of the transgene. The conditions used for PCR were as described in FIGURE 4.2.2 i) PCR product was visualised by running a 5 µl aliquot of each reaction on a 2 % TBE buffered agarose gel and staining with ethidium bromide.The contents of each lane were as follows.

lane 1: Hpa digested pUC low molecular weight markers

lane 2: no DNA

lane 3: non-transgenic rat

lane 4: P6.3

lane 5: P6.3.2

lane 6: P6.3.3

lane 7: P6.3.4

lane 8: P6.3.5

lane 9: P6.3.6

lane 10: P6.3.7

lane 11: P6.3.8

lane 12: P6.3.9

lane 13: P6.3.10

lane 14: P6.3.11

lane 15: P6.3.12

lane 16: P6.3.13

PCR Identification of F1 generation PEPCK-IGF-I Transgenic Rats

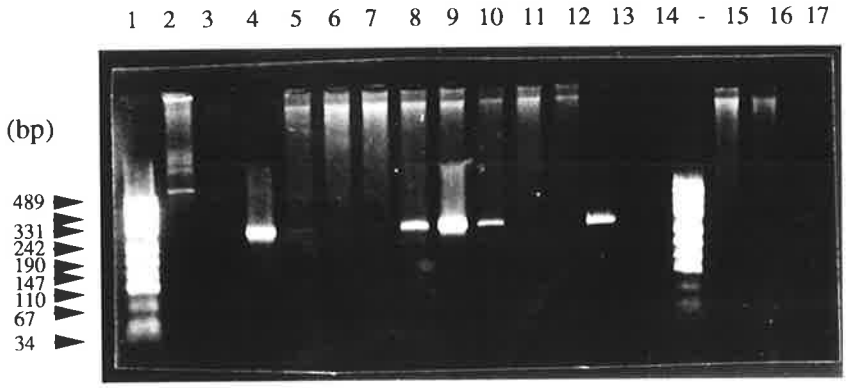


FIGURE 4.2 8 ii) Southern analysis was carried out on the F1 generation of P6.3 using Bam HI. 20 μg of DNA from each animal was digested overnight before being run on a 0.8 % agarose gel. The gel was subsequently transferred to Nytran™ membrane and the DNA crosslinked to the membrane by UV irradiation. The filter was probed with the oligolabelled Bam HI/Bgl II PEPCK promoter fragment. and was washed up to 65°C in 0.1 x SSC/0.1 % SDS for 30 minutes before autoradiography for 72 hours. It should be noted that ethidium bromide staining of the gel prior to transfer revealed that samples P6.3.6 and P6.3.10 appeared to have much more (>20 μg) DNA than other lanes. The sizes of co-electrophoresed SPP1 markers are given on the left hand side.

lane 1: non-transgenic rat

lane 2: P6.3

lane 3: P6.3.2

lane 4: P6.3.5

lane 5: P6.3.6

lane 6: P6.3.7

lane 7:.P6.3.8

lane 8: P6.3.10

lane 9: P6.3.11

lane 10: P6.3.13

Southern Analysis of PEPCK-IGF-1a) Rats

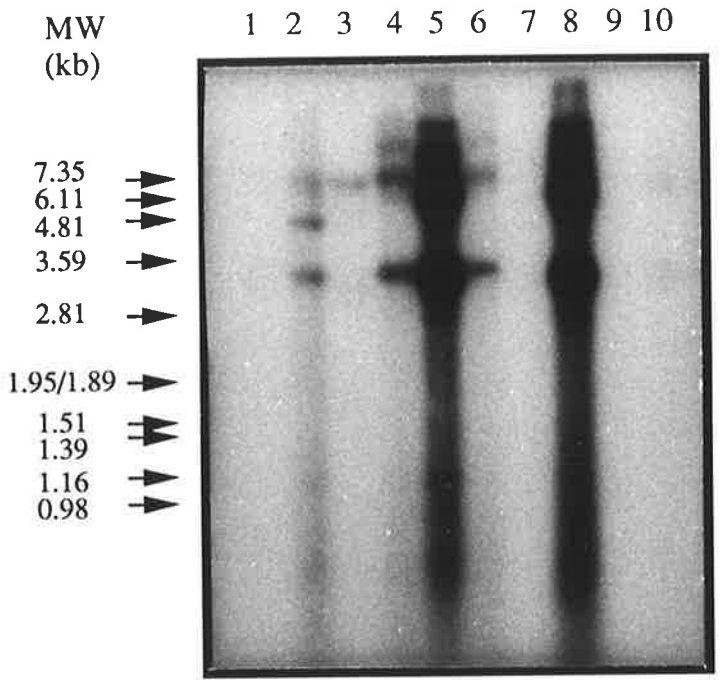


FIGURE 4.2.9 i) A ribonuclease protection assay was carried out on 20 μg (except where stated) of total liver RNA from a number of PEPCK-IGF-Ia) mice (#4,6,11,12,13,14,18,20,21,and 25) and rats (P1.1 and P6.3). The probe used corresponded to 3' end of the hIGF-I cDNA and its mature size was 159 bps. This probe should protect 102 bp of the PEPCK-IGF-Ia) transgene mRNA. Hybridisation of probe to RNA was carried out overnight at 50°C. Digestion of hybrids was with both RNase A and T1 at 3 °C for 30 minutes. Hybrids were precipitated and run on a 6 % polyacrylamide gel before autoradiography for 14 days. The size of co-electrophoresed Hpa II digested pUC low molecular weight DNA markers is given on the left of the figure (lane 1)

lane 2: Probe without digestion

lane 3: 5 μg RNA from hIGF-I expressing CHO cell line

lane 4: 5 μg RNA from hIGF-I expressing CHO cell line

lane 5: liver mouse #4

lane 6: liver mouse #9

lane 7: liver #11

lane 8:.liver #12

lane 9.liver #13

lane 10: liver #14

lane 11: liver #18

lane 12: liver #20

lane 13: liver #21

lane 14: liver #25

lane 15: liver P1.1

lane 16: liver P6.3

Expression of hIGF-I mRNA in PEPCK-IGF-Ia) Transgenic Mice and Rats

i)

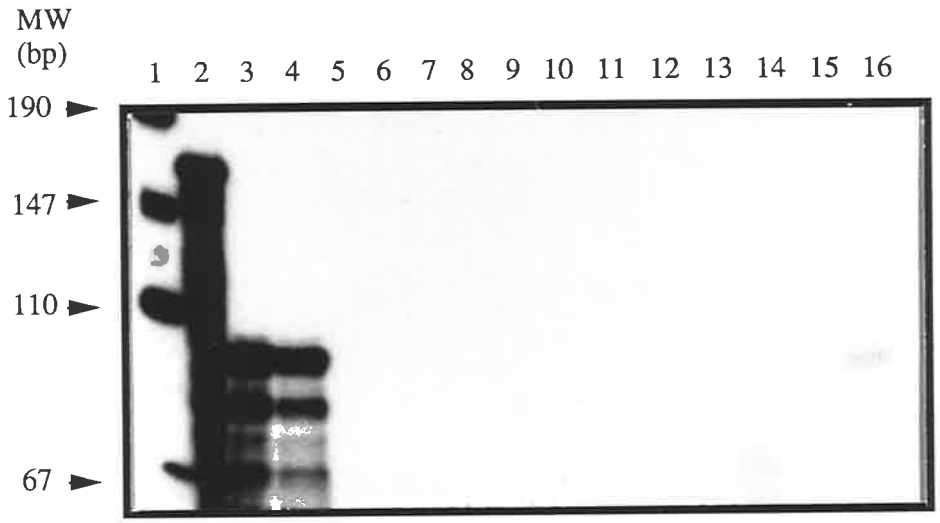


FIGURE 4.2.9 ii) Ribonuclease protection was carried out on 40 μg of total RNA from liver kidney and intestine of rat P6.3 with the hIGF-I 3' riboprobe. RNA was extracted following an overnight fast. The reactions were carried out as described in FIGURE 4.2.9 i) and protected products were separated on a 6 % polyacrylamide gel. Autoradiography was for 72 hours.

lane 1: Hpa II digested pUC markers (sizes given)
lane 2: undigested probe (159 bp)
lane 3: hIGF-I expressing CHO cell line (5 μg RNA)
lane 4: P1.1 liver RNA (40 μg)
lane 5: P6.3 kidney RNA (40 μg)
lane 6: P6.3 liver RNA (40 μg)
lane 7: P6.3 intestine RNA (40 μg)
lane 8: P2.5 liver RNA (40 μg)
lane 9: P2.5 kidney RNA (40 μg)

FIGURE 4.2.9 iv) Ribonuclease protection was performed on total RNA (50 μg) derived from the tissues of F1 PEPCK-IGF-Ia) rat, P6.3.5, and a non-transgenic littermate, P6.3.8, using the 159 bp hIGF-I 3' riboprobe. The assay was carried out as described in FIGURE 4.2.9 i) and the protected species were analysed on a 6 % polyacrylamide gel which was autoradiographed for 24 hours. RNA extracted from tissues of P6.3 was included as a positive control. Only the 102 bp protected species are shown. Autoradiography was for 72 hrs.

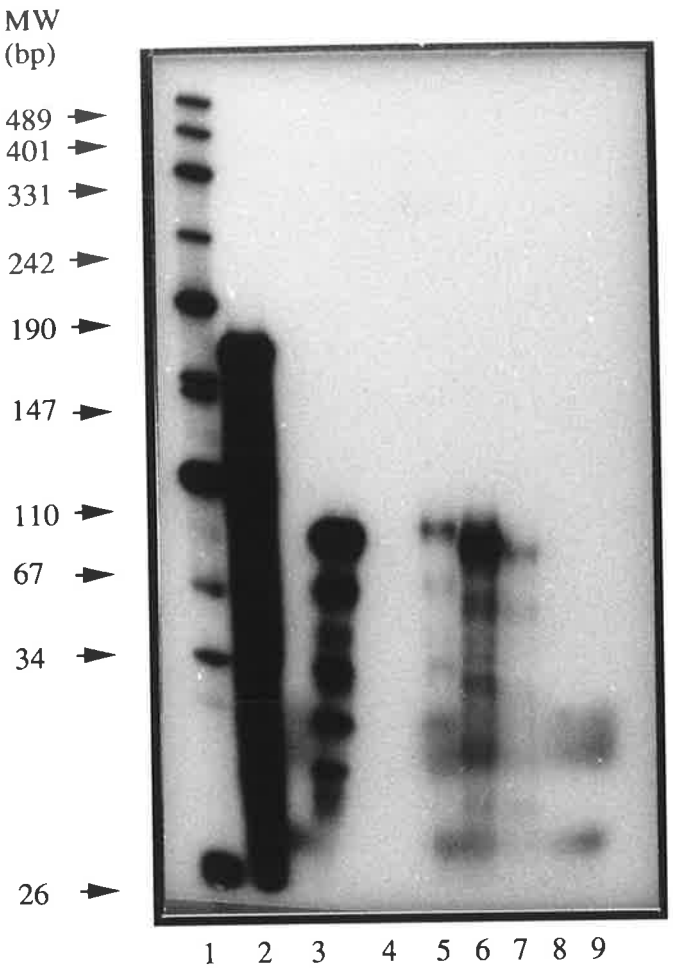
lane 1: non-transgenic rat liver
lane 2:P6.3 liver
lane 3: P6.3.kidney
lane 4: P6.3 intestine
lane5: P6.3.5 liver
lane 6: P6.3.5 kidney
lane 7: P6.3.5 intestine
lane 8: P6.3.8 liver
lane 9: P6.3.8 kidney
lane 10: P6.3.8 intestine
lane 11: non-transgenic rat kidney

FIGURE 4.2.9 v) Ribonuclease protection was performed on 50 μg of total RNA from PEPCK-IGF-Ia) F2 generation animals, P6.3.5.4, P6.3.5.5 and P6.3.5.6 using the hIGF-I 3' riboprobe previously described. The protected species were separated on a 6 % polyacrylamide gel and visualised by autoradiography for 5 days.

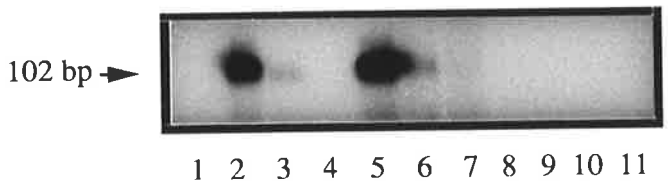
lane 1: P6.3.5.4 liver
lane 2: P6.3.5.4 kidney
lane 3: P6.3.5.4 intestine
lane 4: P6.3.5.4 heart
lane 5: P6.3.5.5 liver
lane 6: P6.3.5.5 kidney
lane 7: P6.3.5.5 intestine
lane 8: P6.3.5.5 heart
lane 9: P6.3.5.6 kidney
lane 10: P6.3.5.6 liver
lane 11: P6.3.5.6 intestine
lane 12: P6.3.5.6 heart

Expression of The PEPCK-IGF-Ia) Transgene In Rats

ii)



iv)



v)

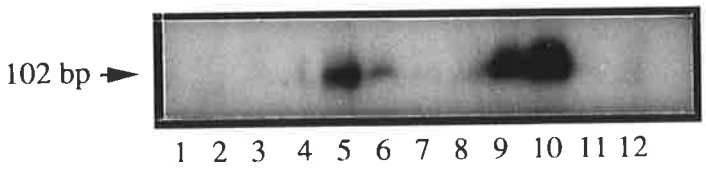


FIGURE 4.2.9 iii) Rats were fasted for 24 hours before blood was taken from each animal at sacrifice. Plasma (50 %) was chromatographed by gel filtration under acidic conditions to separate IGFBPs from IGF-I. Fractions were collected (0.25 ml) and 50 μl aliquots of each fraction were assayed in triplicate using a monoclonal antibody radioimmunoassay (Tomas et al., 1992). The elution position of hIGF-I was verified as being between 9-10 mls by chromatographing radiolabelled hIGF-I on the column before and after the plasma samples. The concentration of hIGF-I present in P6.3 was determined by comparison with hIGF-I standards included in the assay. The large peak of immunoreactivity eluting at 7-8 ml is due to interference in the assay by IGFBP.

Acid gel filtration and assay of PEPCK-IGF-Ia) rat plasma

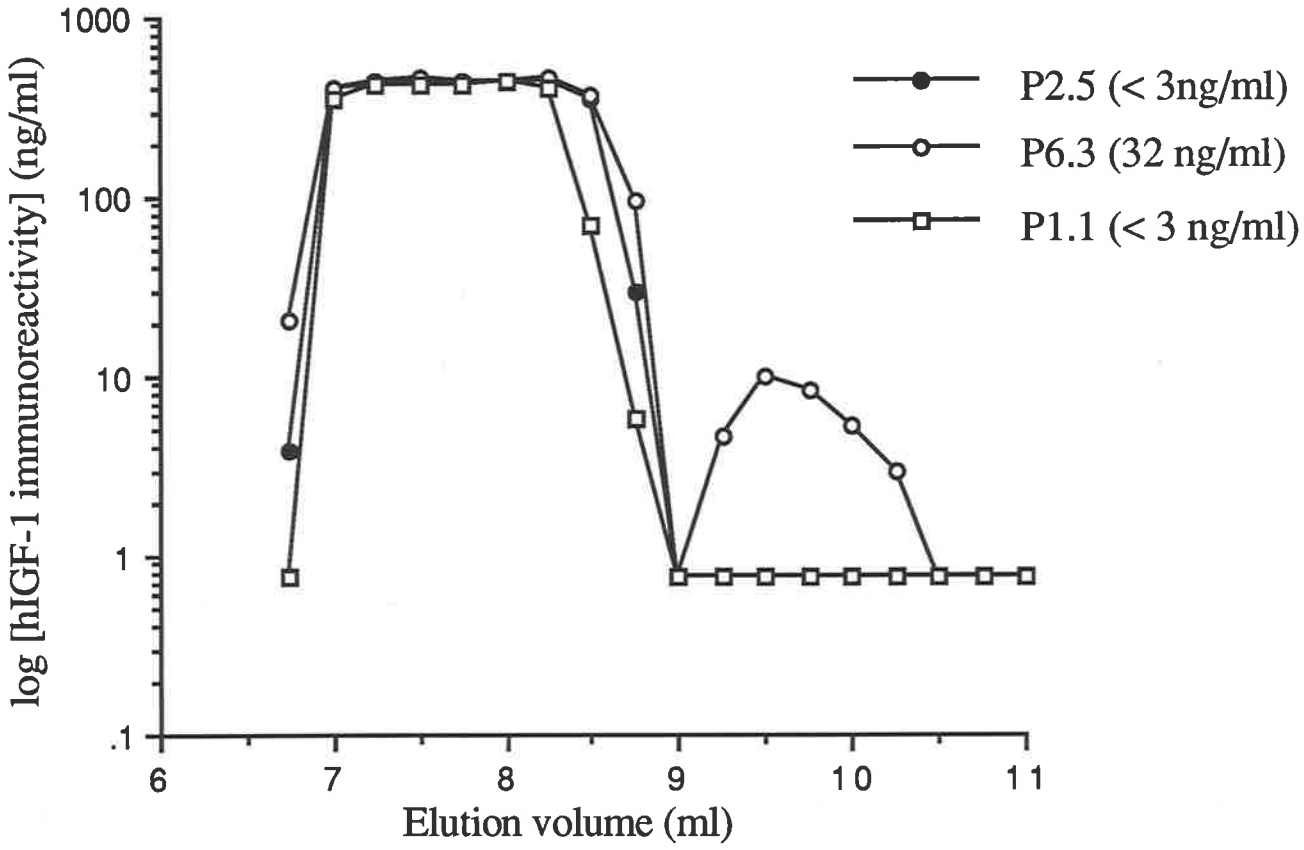


FIGURE 4.2.10 F1 generation PEPCK-IGF-Ia) rats were fasted for 24 hours and then refed ad libitum. Blood samples were taken after fasting and following re-feeding. Plasma samples were diluted and denatured by heating before the equivalent of 2 µl of the original plasma was electrophoresed on a 12.5 % SDS polyacrylamide gel under non-reducing conditions. The gel was subsequently electroblotted on to a nitrocellulose filter and allowed to air dry before being probed with ^{125}I-IGF-II tracer overnight. Following washing of the filter to eliminate background hybridisation the filter was autoradiographed for 14 days. The size of ^{14}C molecular weight protein markers are shown on the left of the autoradiograph. The samples loaded were as below, where (f)=plasma during fast and (rf)= plasma after re-feeding.

lane 1: P6.3.5 (f)

lane 2: P6.3.5 (rf)

lane 3: P6.3.6 (f)

lane 4: P6.3.6 (rf)

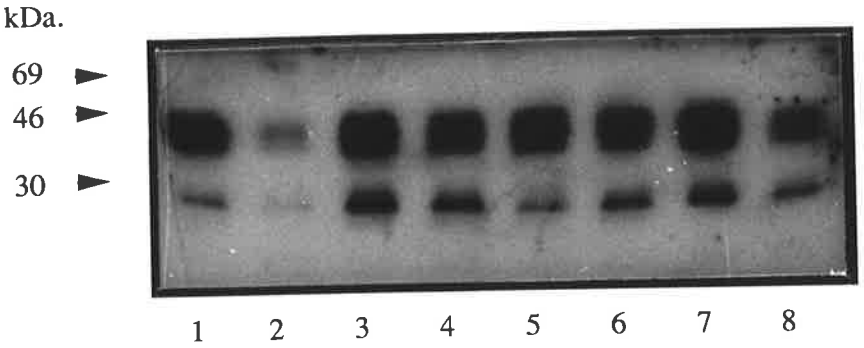
lane 5: P6.3.7 (f)

lane 6: P6.3.7 (rf)

lane 7: P6.3.8 (f)

lane 8: P6.3.8 (rf)

Production of IGFBPs by PEPCK-IGF-Ia) Transgenic Rats



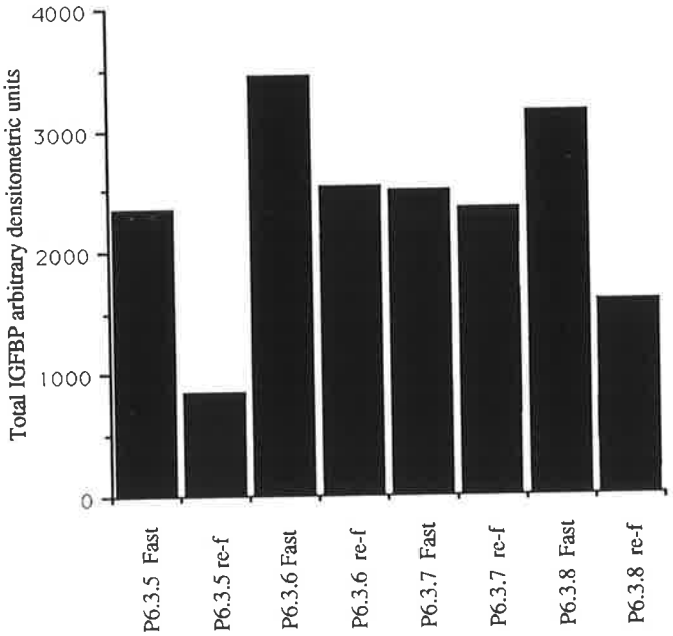


FIGURE 4.2.11 i) The Polymerase Chain Reaction was carried out on genomic DNA from the tails of PEPCK-IGF-Ia) F2 generation animals (offspring of P6.3.5). The primers were as previously described (figure 4.2.2. i)), as were the reaction conditions. Aliquots of each reaction were electrophoresed on 2 % polyacrylamide gels and the 285 bp product visualised by ethidium bromide staining of the gels.

lane 1: Hpa II digested pUC low molecular weight DNA markers

lane 2: no DNA

lane 3: non-transgenic rat

lane 4: non-transgenic rat

lane 5: P6.3.5.1

lane 6: P6.3.5.2

lane 7: P6.3.5.3

lane 8: P6.3.5.4

lane 9: P6.3.5.5

lane 10: P6.3.5.6

lane 11: P6.3.5.7

lane 12: P6.3.5.8

lane 13: P6.3.5.9

lane 14: Markers

lane 15: P6.3.5.10

lane 16: P6.3.5.11

lane 17: P6.3.5.12

lane 18: P6.3.5.13

PCR Identification of F2 generation PEPCK-IGF-I Transgenic Rats

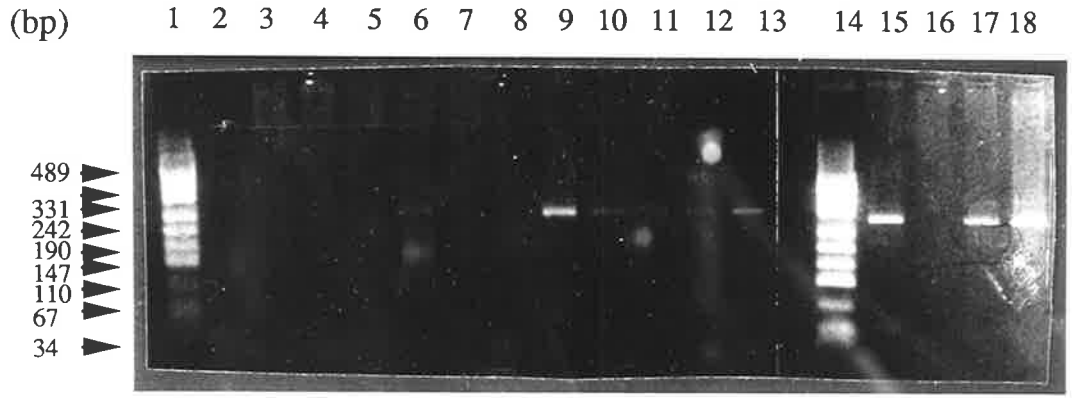


FIGURE 4.2.11 ii) Southern analysis was carried out on the F2 generation PEPCK-IGF-Ia) rats (offspring of P6.3.5 and P6.3.7) . Genomic DNA (20 μg) was spermine precipitated and digested to completion with Bam HI. Samples were electrophoresed on 0.8 % TBE buffered, agarose gels and the gels transferred to Nytran™ membranes. DNA was crosslinked by UV irradiation to the filters which were probed with the Bam HI/Bgl II PEPCK promoter fragment before being washed at 60 °C for 45 minutes in 0.1 x SSC/0.1 % SDS and autoradiographed for 48 hours. Included on this autoradiograph as a positive control was DNA from the F1 generation parents of these F2 animals. The molecular weight of co-electrophoresed SPP1 DNA markers are shown on the left of the autoradiograph.

lane a: P6.3.5 lane b: P6.3.6 lane c: P6.3.7

lane d: P6.3.7.1 lane e: P6.3.7.2 lane f: P6.3.7.3

lane g: P6.3.7.4 lane h: P6.3.7.5 lane i: P6.3.7.6

lane j: P6.3.7.7 lane k; P6.3.7.8 lane l: P6.3.7.9

lane m: P6.3.7.10 lane n: P6.3.5.3 lane o: P6.3.5 4

lane p: P6.3.5.5 lane q: P6.3.5.6 lane r: non-transgenic rat

FIGURE 4.2.11 iii) Bam HI Southern analysis was carried out as above on the progeny of P6.3.6. This filter was exposed to super sensitive (Kodak X-Omat) film for 7 days.

lane a: P6.3.6.1

lane b: P6.3.6.2

lane c: P6.3.6.3

lane d: P6.3.6 4

lane e: P6.3.6.5

lane f: P6.3.6.6

lane g: P6.3.6.7

lane h: P6.3.6.8

lane i: P6.3.6.9

lane j: P6.3.6.10

lane k: P6.3.6.11

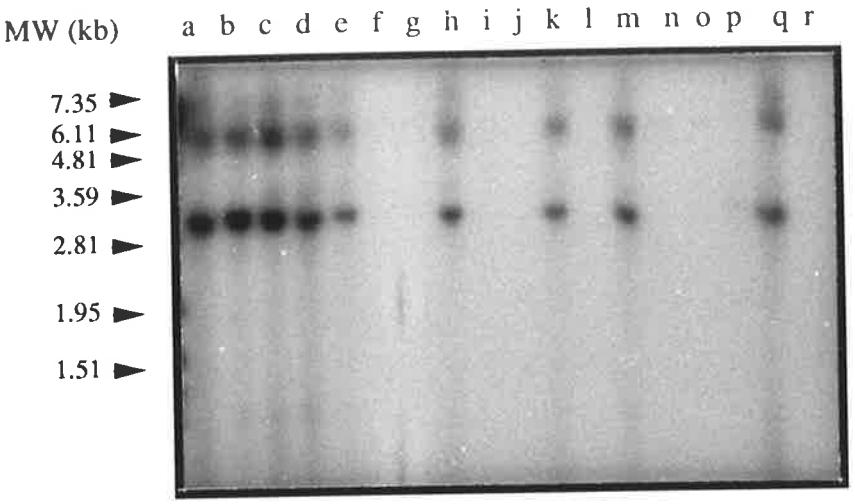
lane l: P6.3.6.12

lane m: P6.3.6.13

lane n: non-transgenic rat

Bam HI Southern Analysis of PEPCK-IGF-I
F2 Generation Animals

ii)



iii)

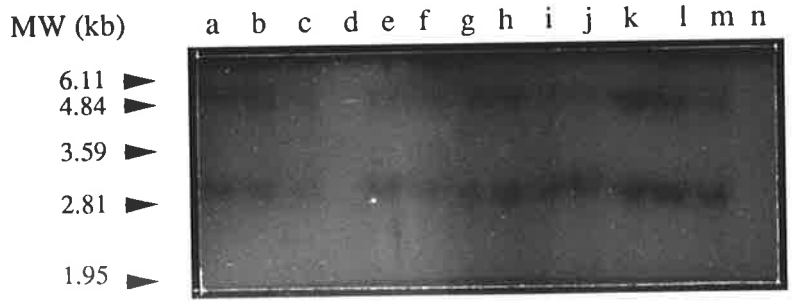
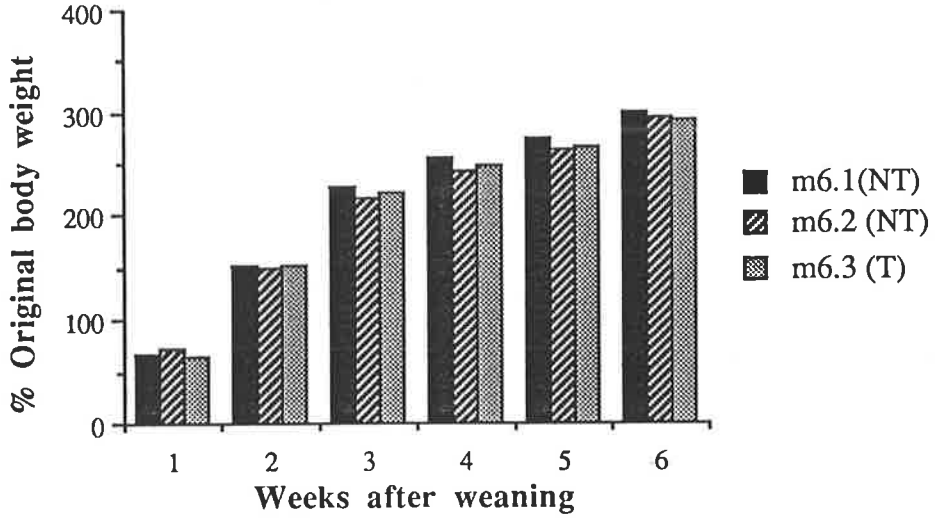


FIGURE 4.2.12 i) The growth of three male PEPCK-IGF-Ia) microinjected rats from weaning. Weights are expressed as a percentage of the original body weight at weaning. (T)=transgenic and (NT)=non-transgenic.

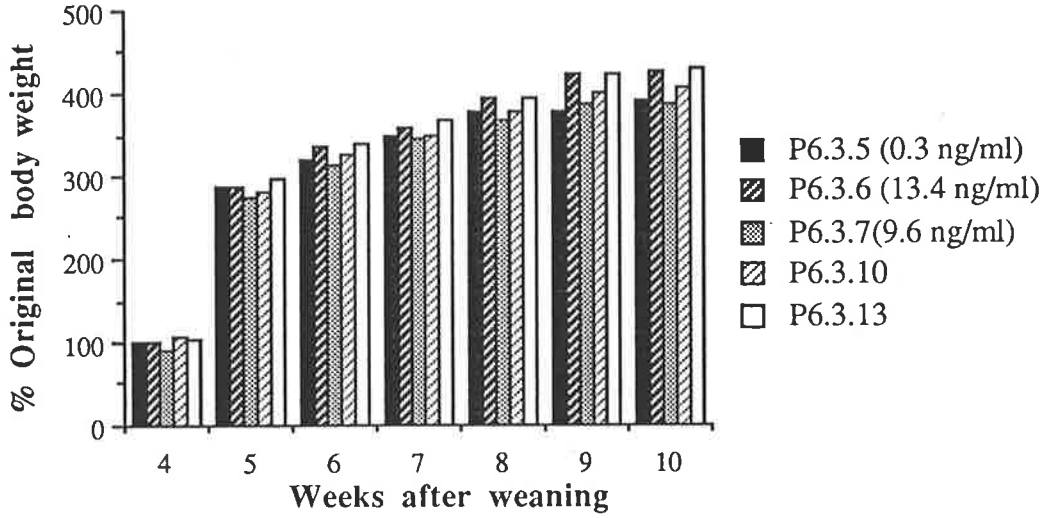
FIGURE 4.2.12 ii) The growth of expressing PEPCK-IGF-Ia) transgenic, F1 generation, male rats compared to their age matched non-expressing littermates. Weights have again been expressed as a percentage of original weight at weaning. The numbers in brackets indicate the plasma levels of hIGF-I detected during feeding using a monoclonal radioimmunoassay (Tòmas et al., 1992).

FIGURE 4.2.12 iii) The growth of F2 generation transgenic PEPCK-IGF-Ia) male rats (T) compared to that of non-transgenic littermates (NT). Weights are expressed as a percentage of original weight at weaning.

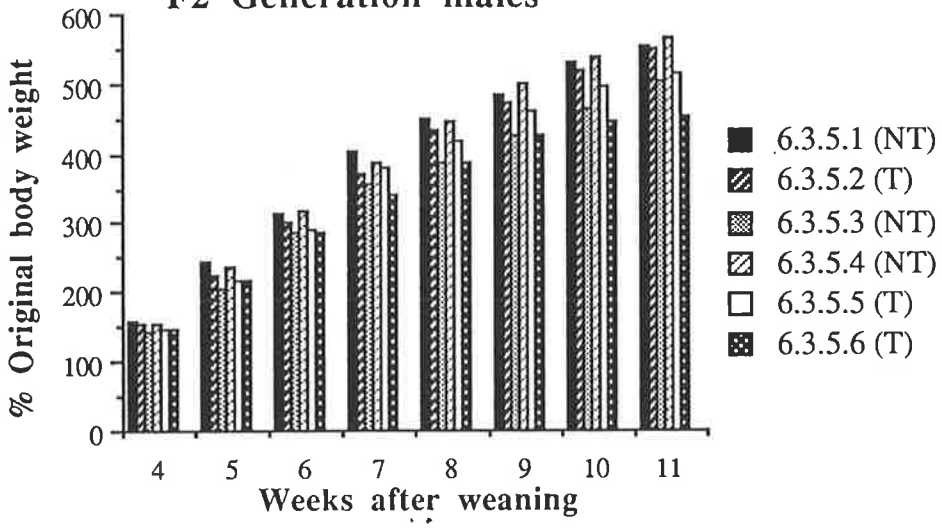
Growth of PEPCK-IGF-Ia) Microinjected Rats



Growth of PEPCK-IGF-Ia) Transgenic Male Rats



Growth of PEPCK-IGF-Ia) P6.3.5, F2 Generation males



4.3 DISCUSSION AND FUTURE EXPERIMENTS:

This study has shown the PEPCK-IGF-Ia) transgene to be integrated at similar efficiencies into the germ-line of both mice and rats. The 42 % integration efficiency of the construct into rats is somewhat below that reported for integration of the mouse Ren-2 transgene into transgenic rats (Mullins et al., 1990) and greater than the 33 % and 29 % integration efficiencies achieved with the human Renin and Angiotensin genes respectively, in the same species (Ganten et al., 1992). Furthermore, this efficiency is consistent with the 56 % incorporation of the MTIIa-pGH construct into rats observed in my earlier studies. Hence, this study provides further evidence of the feasibility of transgenesis in rats.

Despite incorporation of the PEPCK-IGF-Ia) transgene into 49 % of mice born, no expression of the transgene was observed in any of these animals. Southern analysis on two of these non-expressors (figure 4.2.3) suggests that the transgene had not undergone rearrangement during its integration into the genome. Therefore, the lack of expression in these individuals is likely to be related to the site of genomic integration ie. a "Position effect."(Lima-de-Faria, A. 1983; Jaenisch,R. 1988). However, I cannot rule out the possibility of transgene rearrangement in the other non-expressing mice. As analysis of the transgene mRNA was only made for 12/27 of the transgenic mice, I cannot exclude the expression of the transgene mRNA in the other transgenics. However mRNA production could not have led to the production of protein, as RIA of all mouse plasma samples revealed hIGF-I was undetectable.

Importantly, unlike the situation in the mice, expression of the PEPCK-IGF-Ia) transgene was observed in 1/11 (9 %) of the primary transgenic rats. Evidence from DNA dot blots (figure 4.2.1) suggested that in some animals, transgene sequences may have been lost during integration. This deletion of important transgene sequences may be responsible for the non-expression observed in these particular animals. The expression observed in rat P6.3 was detected following an overnight fast, as expected for expression controlled by the PEPCK promoter (Hopgood et al., 1973). The expression of hIGF-I was relatively low in this animal, (32 ng/ml) compared with the expression of endogenous IGF-I in the rat (Daughaday et al., 1989). It is difficult to compare the expression achieved in this individual animal with that achieved in MT-IGF-I transgenic mice produced by Mathews et al., (1988b), due to their use of an antibody which did not distinguish transgene-derived IGF-I from endogenous mIGF-I. Nonetheless, the 32 ng/ml produced by P6.3, is

consistent with the levels of bGH expression achieved in certain lines of animals transgenic for bGH under the control of the same PEPCK promoter region used in this study (McGrane et al., 1988,1990). These authors also obtained lines of mice expressing up to 2000 ng/ml of bGH, implying that the PEPCK promoter is indeed capable of directing higher level *in vivo* expression. Whether such levels of expression are attainable using the PEPCK-IGF-Ia) construct will require further production of transgenic animals with this construct.

Expression of hIGF-I was detected predominantly in the liver and kidneys of the expressing animal following fasting while lower levels of mRNA were found in the intestine of this animal. A similar pattern of expression was observed by McGrane et al. (1988), in PEPCK-bGH transgenic mice, indicating that the 460 bp region of the PEPCK promoter used in these studies contains sequences required for the liver/kidney specific expression of transgenes. Furthermore, the expression pattern observed suggests that the chromosomal integration site of the PEPCK-IGF-Ia) transgene in rat P6.3 does not interfere with tissue-specific expression of the transgene. Predominant liver expression of hIGF-I mRNA implies that the plasma levels of hIGF-I detected in this animal probably account for the majority of total transgene expression.

The PEPCK-IGF-Ia) transgene has been successfully transmitted through two generations of animals from Founder P6.3 (see figures 4.2.8 i) and 4.2.11 ii)-iii)) In the F1 generation the transgene was inherited by 50 % of the offspring in the predicted Mendelian fashion. However in one of the F2 generation litters, the transgene was inherited by a disproportionately large number of animals (figure 4.2.11 ii)). The reason for this abnormal segregation of the transgene in unknown. Similar distorted ratios of transgene inheritance have been previously reported (Palmiter et al., 1982). Further breeding of animals within this family should enable the inheritance pattern to be more firmly established.

Expression of the transgene has also been inherited through at least one generation. Interestingly, not all F1 animals carrying the transgene were found to be expressing hIGF-I despite the apparent inheritance of the same transgene organisation in each case. Also, the levels of expression amongst the progeny varied. Similar variable patterns of transgene expression within single families of transgenic mice have previously been reported in the literature (Palmiter et al., 1982,1984; McGrane et al., 1988).

In the F1 offspring produced in this study, inheritance of expression appears to be related to sex. Only F1 males which inherited the transgene were seen to be expressing

hIGF-I. Although this phenomena has not yet been verified for expression of the transgene in the F2 generation offspring, it is tempting to suggest that the PEPCK-IGF-Ia) transgene in this family may have become imprinted (for review, see Reik, 1992). As DNA methylation is believed to be associated with this phenomena, further investigation could involve examining the methylation status of the transgene amongst the expressing and non-expressing animals within this family. Such analysis was carried out by McGrane et al., (1988) and confirmed that the methylation status of their PEPCK-bGH transgene did not correlate with the inheritance of expression . Hence, in my studies also, the possibility of another differentially segregating locus which affects the PEPCK promoter cannot be excluded. Further lines of PEPCK-IGF-Ia) transgenic rats will be required before such questions can begin to be answered.

The expression of the transgene observed in two of the F1 generation progeny (P6.3.5, P6.3.7) was decreased when the animals were re-fed following an overnight fast, consistent with the behaviour of the endogenous PEPCK gene (Hopgood et al., 1973; Cimbala et al., 1982). In addition, the tissue specificity of hIGF-I mRNA expression in one of these F1 offspring, has been shown to mimic that of its transgenic parent, implying control of hIGF-I expression by the PEPCK promoter is heritable. To fully examine the control of hIGF-I production in these rats it will be necessary to measure the production of hIGF-I mRNA in these animals under "fed" conditions.

The growth observed in rats expressing the PEPCK-IGF-Ia) transgene was not significantly different from that of control animals suggesting that the amount of hIGF-I produced under normal ad libitum feeding of the animals may not have been biologically significant. Several studies have examined the effect of administered hIGF-I to rats. Schoenle et al. (1985) showed that administration of hIGF-I to hypophysectomised rats for a 6 day period increased both tibial epiphyseal width, and thymidine incorporation into costal cartilage at plasma levels between 168-268 ng/ml. The higher plasma level of hIGF-I also produced an increase in body weight over the 6 day period compared to controls. Guler et al., (1988) have also shown similar body weight increases in hypophysectomised rats in which administration of hIGF-I led to plasma levels of 300-350 ng/ml. More recently, Tomas et al , (1992) have shown significant body weight increases in dexamethasone infused rats, attaining plasma levels of between 700-1000 ng/ml hIGF-I. Thus, although these studies were carried out acutely and have used animals with hormonal pertubations, precluding direct comparison with my studies, the levels of hIGF-I achieved in attaining

increased growth were far in excess of the levels produced in the PEPCK-IGF-I a) transgenic rats. Furthermore, in the single study in which hIGF-I has been administered to intact, normally growing male rats, the observed increases in growth were shown to correspond with increases in plasma IGF-I levels of approximately 200 ng/ml (Hizuka et al , 1986), well above the levels achieved in the PEPCKIGF-I a) expressing rats.

MT-IGF-I, transgenic mice produced by Mathews et al. (1988a), manifested a 1.3-fold increase in weight relative to their non-transgenic littermates. However, in these mice increased plasma IGF-I levels were accompanied by an increase in the levels of plasma IGFBP-3 , the major carrier of circulating IGF-I. By contrast, expression of hIGF-I in the PEPCK-IGF-Ia) rats does not appear to lead to a marked alteration in IGFBP-3 (figure 4.2.10) under fed conditions. It is therefore plausible that the PEPCK-IGF-Ia) rats fail to exhibit increased growth even in the presence of hIGF-I produced basally from the PEPCK promoter, due to clearance of this hIGF-I from the circulation in the absence of sufficient IGFBP-3.

In summary, this study has further confirmed the feasibility of gene transfer to rats. In addition , we have produced a single line of rats which express hIGF-I in a manner which is consistent with control by the PEPCK promoter. These animals appear to express a basal level of hIGF-I which is increased during fasting. Expression of the transgene is predominant in the livers and kidneys of these animals. Despite the presence of extra hIGF-I in the plasma of these rats their growth is normal compared to non-transgenic littermates.

Future work on this line of rats will involve examination of the effect of the transgene derived hIGF-I under conditions of dietary hardship or hormonal manipulation. Specifically, it will be interesting to examine hIGF-I expression in these animals when fed both low carbohydrate/high protein and high carbohydrate/low protein diets (or when starved for longer periods). Although, little effect on the growth of these animals was observed under fed conditions it would be interesting to see whether expression of additional hIGF-I for prolonged periods would enable those animals expressing the transgene to maintain their body weights ahead of the non-transgenics. O'Sullivan et al. (1989) showed that administration of IGF-I to mice during starvation (for 36 hrs) was able to reduce the weight loss in these animals. Furthermore, total body weight may be an inappropriate index of growth in these animals. Mathews et al., (1988a) observed marked specific organomegaly in transgenic mice expressing hIGF-I under the control of the MT-I promoter without an apparent increase in skeletal growth. Therefore, it will be important to examine

the effect of hIGF-I expression in the PEPCK-IGF-Ia) transgenic rats, on carcass composition and individual organ weights. In respect to this, it has recently been shown that IGF-I transgenic mice show a markedly increased brain growth and myelination compared to normal mice, suggesting an important role for this peptide in brain growth and development (Carson et al., 1993).

It would also be beneficial to examine in greater detail the inducibility of the transgene in the available line of rats. This could be done by administration of the glucocorticoid dexamethasone to the animals and measuring changes in the plasma levels of hIGF-I in response to this known inducer of PEPCK transcription (Nechushtan et al., 1987; Magnuson et al., 1987). Also, dibutyryl-cAMP could be administered, as it has been shown to induce expression of PEPCK controlled transgenes when administered to transgenic mice (McGrane et al., 1988). Integral to such studies would be the paralell creation of additional transgenic rat lines using the PEPCK-IGF-Ia) construct. Extra lines of animals would allow better assessment of the potential for higher level expression of the hIGF-I cDNA. Nevertheless, even with only one line of PEPCK-IGF-I animals available we are now in a position to begin to assess the ability of hIGF-I to combat stress-associated nitrogen loss in these animals.

CHAPTER 5
GENERAL DISCUSSION

CHAPTER 5:

GENERAL DISCUSSION

Since the commencement of this project the use of rats in the field of transgenesis has become more widespread, with transgenic rat models for both hypertension and spontaneous inflammatory disease being developed (Mullins et al., 1990; Hammer et al., 1990). However the limited success of rat transgenesis reported prior to my commencement suggested it was necessary initially to establish the procedure in rats using an already proven transgene. Therefore a construct containing the hMTIIa promoter linked to a pGH cDNA was introduced into the rat zygotes by microinjection. This construct was chosen as it had previously been proven to integrate successfully and express biologically active pGH in transgenic mice and pigs (Owens et al. ,1989; Vize et al., 1988). Consistent with the success in these species, a high integration frequency of 56 % was observed in rats. Furthermore, a large proportion of these transgene positive animals (approximately 28 %) were found to have immunoreactive pGH in their blood at the time of weaning. The level of plasma pGH in individual animals varied from as low as 18 ng/ml to as high as 1200 ng/ml and was not related to the number of copies of the transgene. When expression of pGH mRNA was examined in founder rats using a ribonuclease protection assay, the pGH mRNA was detected in a number of tissues ie. liver, kidney, lung, spleen, brain, intestine and heart, consistent with the expression of the metallothionein genes in most adult cell types (Searle et al., 1984). The pattern, tissue distribution and abundance of pGH mRNA varied between individual rats. However, in all but one animal examined the predominant expression of pGH was in the liver. The exception showed greater pGH mRNA in the intestine than in the liver. These findings are in general agreement with the results of transgenesis using the MT-I and MTII promoters in transgenic mice (Palmiter et al., 1986) and strongly suggest that expression of this transgene is heavily influenced by its site of integration. Thus the DNA surrounding the transgene can directly influence the level of pGH expressed and its pattern of expression . Although it is assumed that the transgene has integrated into different regions of the rat genome in each transgenic animal, confirmation should be made using chromosomal *in situ* hybridisation (Lacy et al., 1983) employing the hMTIIa promoter Hind III/Ava I probe.

Recently Palmiter et al., (1993) have identified DNA regions far upstream and downstream of the mouse MT-I and MT-II genes respectively, which are able to confer

position-independent expression on a variety of reporter constructs in transgenic mice. These regions also increased the *in vivo* expression of intronless copies of the rGH gene. The sequences corresponded to sites of DNAse hypersensitivity and are believed to function as Locus Control Regions (LCRs) for the metallothionein genes. Thus, inclusion of such regions (or regions derived from the human MT genes) in any future MTIIa-pGH constructs may well enable consistent expression of the construct in individual transgenic rats.

I have shown the MTIIa-pGH transgene to be heritable in two separate lines of rats. These lines of animals show marked differences in both the levels of blood borne pGH and their growth response. In the D line, transgenic animals exhibit low levels of plasma pGH which have been shown in preliminary experiments to be inducible by zinc. Growth in these animals is not significantly different from non-transgenic controls. In contrast the line derived from M5.5, plasma levels of pGH are high, non-inducible and accelerated growth can be observed from as early as three weeks of age. These results again emphasise the influence which may be exerted by the chromosomal site of integration on the expression and regulation of the MTIIa-pGH transgene.

A number of the founder animals showed increased growth from the time of weaning, however the growth was not directly related to the plasma levels of pGH at this time. While female animals C, M2.1 and M5.5 showed marked growth with plasma pGH levels of 800 ng/ml, 1170 ng/ml and 42 ng/ml respectively, male transgenics such as M5.2 showed little increase in growth despite a plasma pGH level of 360 ng/ml (table 2.2.4). Thus, although pGH stimulated growth in a number of animals not all were equally responsive. This is perhaps not surprising as the growth of these animals would also be dependent on the levels of rat GH present. It is possible that the tissue site of expression may influence the growth response. Thus, while all of the fast growing animals examined showed predominant liver expression the only unresponsive animal examined , Founder D showed high intestinal expression. It is obvious from these anomalies that their are inherent difficulties in studying the growth of founder animals, largely due to differences in the integration site of the transgene.

At the present time it is not known whether the transgene derived pGH directly acts on the rat GH receptors or whether enhanced growth is manifested as a result of displacement of rGH from circulating GH BP and its subsequent action on GH receptors. Hence future studies to elucidate the mechanism of growth in the MTIIa-pGH rats will need to carefully examine the levels of endogenous hormones and binding proteins in these rats eg

rGH (GH/BP) , rIGF-I (IGFBPs) and their possible relationships to the growth observed. Furthermore, the contribution to the growth of these rats by individual tissues and organs needs to be clarified. While meaningful examinations of these areas could not be performed on the founder animals generated in this study, the production of two stable lines of transgenic rats should enable well controlled and detailed experiments to now be performed. Further characterisation of these lines of rats with respect to the onset and distribution of GH expression, its possible regulation by zinc and the subsequent timing of the growth response should ensure that these animals become useful tools to study GH regulated somatic growth.

Before attempting transgenesis in rats using the PEPCK-IGF-I construct it was highly desirable to test for its expression *in vitro*. Therefore the construct was transfected into both CHO cells and H4IIE hepatoma cells by electroporation. It should be noted that expression of constructs *in vitro* rarely reflects expression *in vivo*. While a number of transfectants were isolated, expression of hIGF-I was poor in all but one CHO clone (clone 1O). The poor expression generally observed in the CHO transfectants may be due to the absence of a specific trans-acting factor in this cell line necessary for efficient expression by the PEPCK promoter. In this scenario the single higher expressing clone may have arisen due to fortuitous integration of the transgene into the CHO genome near an enhancer sequence. Further transfections of this construct into CHO cells accompanied by Southern analysis and ribonuclease protection analysis of expression on the resulting transfectants will be necessary to determine whether this is the case. Closer examination of the expression observed in clone 1O could also be informative. In this regard the PEPCK promoter riboprobe could be employed to assess the expression of the transgene mRNA in this cell line and its regulation by additional factors such as glucagon or cAMP

In regard to the low expression observed it may be worth noting that the half-life of endogenous PEPCK mRNA is extremely short (approximately 30 minutes) (Nelson et al., 1980). Although the hGH sequences were included in the construct to increase the stability of the fusion mRNA species, it is possible that the inclusion of additional PEPCK derived sequences 3' of the IGF-I cDNA may have conferred instability to the transgene message.

Expression in clone 1O was lower than that previously observed for the same IGF-I cDNA driven by a retroviral promoter element in CHO cells (McKinnon et al., 1992). The material produced by this clone was shown to be hIGF-I by radioimmunoassay and its bioactivity confirmed in a L6 myoblast protein synthesis assay. Furthermore, this particular clone produced hIGF-I mRNA in a manner consistent with control of expression by the

glucocorticoid-inducible, insulin-repressible PEPCK promoter, suggesting that the construct was functional and could be used for transgenesis studies.

Expression of the PEPCK-IGF-I construct in the H4IIE cell line was very low. Unexpectedly, analysis of the endogenous PEPCK gene in non-transfected H4IIE cells showed that the presence of IGF-I in the culture medium was able to inhibit the production of PEPCK mRNA even in the presence of inducers of PEPCK transcription. This indicated that expression of the transgene could be subject to feedback inhibition by IGF-I, preventing the isolation of clones expressing significant hIGF-I. Hence the H4IIE cell line was unsuitable as an *in vitro* model for studying the regulation of the transgene. If future transfections were to be performed using the PEPCK-IGF-I construct, kidney cell lines would now be the likely choice of cell line for use. Such cell lines express the PEPCK gene, but insulin (and hence IGF-I) is not the dominant influence on transcription in these cells (Inedjian et al. ,1975). Thus, induction of the transfected PEPCK-IGF-I gene by glucocorticoids or cAMP should occur regardless of the accumulation of hIGF-I in the culture medium.

Having shown the potential for the expression of the PEPCK-IGF-I construct in tissue culture, microinjections of the transgene were performed into rat oocytes. Integration of the transgene was shown for 42% of the rats produced, a frequency consistent with the findings for the MTIIa-pGH construct in rats and substantially higher than the 7 % reported recently for integration of a metallothionein-PEPCK transgene in rats (Zajac et al., 1993).

Of the animals carrying the transgene, a single male (P6.3) was found to be expressing hIGF-I. The level detected following fasting in this animal was approximately 34 ng/ml, consistent with the low expression of the construct observed *in vitro*. The transgene was found to be arranged in both head-to tail and tail-to-tail arrays in this animal and this pattern was heritable through two generations, indicating stability of the construct in the germ-line. The inherited transgene was expressed by only 3/6 of the F1 offspring containing the gene indicating that other epigenetic factors (reviewed by Monk, 1990) may control expression of the transgene in this family. In two of the animals, expression was induced by fasting and inhibited by re-feeding suggesting control of hIGF-I by the PEPCK promoter. Furthermore in all expressors examined (Fo, F1 and F2 generations), transgene mRNA has predominated in the liver and kidneys, further evidence for control of expression by the PEPCK promoter. These findings also imply that the site of transgene integration in

this line of rats does not affect the tissue-specific expression of the transgene. This appears to be in contrast with the situation observed for the MTIIa-pGH construct.

Studies have recently shown that there were significant effects on glucose metabolism in mice containing PEPCK-bGH transgenes (Valera et al., 1993). Thus it may be relevant to examine this area in the PEPCK-IGF-I rats, as many of GH's effects are via IGF-I. As with studies I have performed in tissue culture cells, the effect of hIGF-I expression on the endogenous PEPCK gene in these transgenic animals could be examined as a preliminary to examination of gluconeogenesis in these animals.

Future experiments on this line of animals will focus on attempting to induce the expression of hIGF-I to higher levels by administration of synthetic glucocorticoids such as dexamethasone, dibutyryl-cAMP or by feeding the animals a special carbohydrate-free diet. Longer term fasting of the animals is constrained by ethical considerations and is therefore not the most favoured option. Furthermore, a thorough classification of all remaining animals in the pedigree with respect to their expresssion of hIGF-I is planned as is concurrent breeding of expressing F2 animals. As only 26 rats were generated from the PEPCK-IGF-I microinjections, further microinjections of the PEPCK-IGF-I or PEPCK-des(1-3)IGF-I transgenes will be necessary to determine whether higher levels of expression are achievable with these constructs.

McGrane et al., (1990) reported an increased frequency of expression in transgenic mice containing the bGH structural gene linked to greater lengths of PEPCK 5' region than used here (2000 bp compared to 460 bp), suggesting that elements further upstream of the PEPCK gene contribute to its ability to be expressed. This idea has been strengthened by the recent discovery of a LCR-like sequence 4.8 kb upstream of the rat PEPCK gene (Cheyette et al. ,1992). Modification of the existing IGF-I and des(1-3)IGF-I constructs to include such LCR sequences may therefore increase the number of expressing animals and shield the promoter from the influence of the neighbouring DNA in any future transgenic rats. Use of LCR sequences in constructs for transgenesis has also been reported to increase the expression level of intron-less constructs *in vivo* (Palmiter et al., 1993). While the effect on hIGF-I expression of heterologous introns included in the PEPCK-IGF-I construct is not yet known, inclusion of PEPCK LCR-like sequences may alleviate the need for these in future.

The growth rate achieved by the PEPCK-IGF-I transgenic rat line was not significantly different from that of non-transgenic rats under a regime of *ad libitum* feeding.

Under these conditions expression of hIGF-I from the transgene is predicted to be extremely low and thus the essentially normal growth of these animals is not surprising. It should be remembered that MT-IGF-I transgenic mice expressing hIGF-I were only approximately 1/3 larger than their non-transgenic littermates (Mathews et al. ,1988). It will be important to examine the growth characteristics of the existing PEPCK-IGF-I rat line and any additional transgenic PEPCK-IGF-I lines generated, under conditions shown to induce expression of the transgene. It will be necessary to determine organ weights and carcass compositions of these animals to assess the contribution to growth made by various tissues. This may be particularly pertinent in view of the specific organomegaly observed in IGF-I transgenic mice and the role of IGF-I as a autocrine / paracrine regulator of the growth of numerous tissues (Mathews et al., 1988; Quaife et al. ,1989; Carson et al., 1993). Thus, while levels of hIGF-I produced from the liver and released in an endocrine fashion in the PEPCK-IGF-I rats may not be sufficient to stimulate significant skeletal growth, hIGF-I acting locally in individual tissues may facilitate the growth of these tissues.

Although, expression of IGF-I has not been shown in the brain of PEPCK-IGF-I rats, it would be of interest to examine this tissue. Evidence suggests that the more potent des(1-3)IGF-I peptide is formed and acts to stimulate the growth of the brain (Sara et al., 1986). Carson et al (1993) showed that IGF-I produced in the brains of IGF-I transgenic mice stimulated significant growth of this tissue. Thus it is possible that low level expression of a PEPCK-IGF-I transgene in the brain could also show pronounced growth effects in this tissue via the generation of des(1-3)IGF-I.

The importance of IGF-I in mammalian growth has recently been re-affirmed by creating chimaeric mice in which the IGF-I gene has been knocked out by gene targeting in embryonic stem cells (Robertson et al ,1993). The resultant heterozygous mice are 60 % of the size of non-transgenic littermates at birth, do not undergo a pubertal growth spurt and reach only 30 % the size of normal animals at maturity. In addition, both males and females exhibit hypoplasia of the reproductive tract and are sterile. Thus, these studies provide direct evidence that IGF-I plays a role in embryonic growth but its major contribution is to the postnatal growth of mammals. While such experiments will continue to be crucial for determining the role of IGF-I in mammalian development, traditional transgenesis approaches as used in my studies are powerful in testing specific hypotheses of IGF-I action.

It is now becoming possible for researchers to incorporate signal sequences into the genome which allow the removal of intervening sequences by site-specific recombination (Fukushige et al., 1992; Orban et al., 1992; O'Gorman et al., 1991)). These sequences require the presence of an recombinase protein in *trans* to facilitate this process (Baubonis et al., 1993). If such sequences were to be placed by homologous recombination next to a specific target gene, that target gene could be excised. Therefore a very powerful approach for studies of IGF-I action in the future may be to generate a line of animals containing the IGF-I gene (or specific exons) flanked by these signal sequences. A second line of animals could then be produced in which the recombinase protein was expressed under the control of a tissue-specific promoter element. Double transgenic offspring generated as a result of mating these two lines should excise the IGF-I gene (or specific regions) only in those tissues in which the recombinase was expressed. Such experiments will form the main armourmant of molecular biology against the mystery of IGF-I action in the coming years.

In addition to the use of gene targeting and site-specific recombination, the future transgenesis in the IGF-I field may benefit from the advances made in manipulation of yeast artificial chromosome vectors (YACs). YAC vectors are capable of housing extremely large pieces of DNA (over 2 Megabases) (Burke et al., 1987). Recently YACs containing transgenes up to 85 kb have been successfully used to generate transgenic animals (Schedl et al. ,1992, 1993; Choi et al., 1993). Thus the potential now exists to introduce the entire IGF-I gene into transgenic animals eliminating the problems associated with the use of poorly expressed cDNAs.

The experiments described in this thesis demonstrate that the production of transgenic rats for biomedical research is not only feasible but in general more efficient than production of transgenic mice. New lines of rats have been generated in which either pGH or hIGF-I are expressed in different patterns. Although further classification of these animals is required both groups of animals offer the potential for elucidating many of the unsolved questions surrounding the actions of both GH and IGF-I in mammalian growth.

CHAPTER 6
MATERIALS AND METHODS

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MATERIALS AND METHODS
6.1 ABBREVIATIONS

ATP: adenosine triphosphate

APS: ammonium persulphate

BCIG: 5-bromo-4-chloro-3-indolyl-ß-∂-galactopyranoside

bp: base pairs

BSA: Bovine Serum Albumin

CHO: Chinese Hamster Ovary

DNase: deoxyribonuclease

dNTP: deoxynucleoside triphosphate

DMEM: Dulbeccos Modified Eagles Medium

DMSO: Dimethyl sulphoxide

DTE: Dithioerythritol

DTT: Dithiothreitol

EDTA: ethylene diaminetetraacetic acid

FBS: fetal bovine serum

FCS: fetal calf serum

GH: Growth hormone

HEPES: N-2-hydroxyethylpiperazine-n'-2-ethane sulphonic acid

HPLC: high pressure liquid chromatography

IPTG: isopropyl-ß-∂-thio-galactopyranoside

IGF: Insulin-Like Growth Factor

IGFBP: Insulin-like growth factor binding protein

PBS: phosphate-buffered saline

PEG: polyethylene glycol (MW 6000)

RNase: ribonuclease

SDS-PAGE: Sodium dodecyl sulphate polyacrcrylamide gel electrophoresis

Tris: Tris (hydroxymethyl) aminomethane

TCA: trichloroacetic acid

6.2 MATERIALS

6.2.1 Chemicals drugs and general reagents

The following were obtained from Sigma Chemical Co. St. Louis, MO, USA

Acrylamide, agarose (Type 1), ampicillin, ammonium persulphate (APS) bisacrylamide (N,N'-methylene-bis-acrylamide), bovine serum albumin (BSA), chloramphenicol, dexamethazone, dibutyryl cyclic adenosine monophosphate (cAMP) deoxyribonucleotide triphosphates (dNTPs), dithiothreitol (DTT), dithioerythritol (DTE), ethidium bromide, ethylenediaminetetra-acetic acid (EDTA), ethylene glycol-bis (ß-amino-ethyl ether) isopropyl-thiogalactoside (IPTG), guanidinium isothiocyanate, N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES), salmon sperm DNA, sodium dodecyl sulphate (SDS), 2-Mercaptoethanol, spermidine, spermine, Tris base.

Sources of other important reagents were as follows :

5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (BCIG): BRL

Ficoll 400: Pharmacia

phenol: BDH chemicals

polyethylene glycol 6000: BDH chemicals

N,N,N',N'- tetramethethylethenediamine (TEMED): Tokyo Kasei

trichloroacetic acid (TCA): Univar Pty. Ltd.

tRNA, *E. coli:* BRL

glycogen: Boehringer Mannheim.

Kits for dideoxy DNA sequencing, oligo-labelling of DNA, *in vitro* synthesis of RNA, kinasing oligonucleotides, polymerase chain reaction and genecleaning were obtained from Biotechnology Research Enterprises of South Australia Ltd., (Bresatec),Adelaide, South Australia Kits for Ribonuclease protection assays were supplied by Bresatec on licence from Ambion Pty. Ltd. Site-directed mutagenesis kits were purchased from Biorad Australia. All other chemicals and reagents were of analytical grade.

Hanks Balanced Salts and most plasticware used in tissue culture was purchased from Gibco, Grant Island, N.Y., USA.

6.2.2 Enzymes

All restriction enzymes used during the course of this work were purchased from either Pharmacia or Toyobo Inc.

Other enzymes were obtained from the following sources:

Calf intestinal phosphatase (CIP) : Boehringer Mannheim

E. coli DNA polymerase I (Klenow fragment) : Bresatec

lysozyme : Sigma

proteinase K : Boehringer Mannheim

Ribonuclease A (RNase A) : Sigma The stock solution (10mg/ml) was incubated at 100 °C for 10 min to inactivate any DNase activity. Also enzymes for Ribonuclease Protection Assays (RPAs) were supplied by Ambion Pty. Ltd.

Ribonuclease T1 (RNase T1) : Pharmacia and Ambion

T4 DNA ligase : Bresatec.

T4 DNA polymerase : Integrated Sciences Pty. Ltd.

T7 RNA polymerase:Bresatec

SP6 RNA polymerase:Bresatec

6.2.3 Radiochemicals

[α-^{32}P] dATP (1800 Ci/mmol), [α-^{32}P] dCTP (1800 Ci/mmol), [γ-^{32}P] ATP (>2000 Ci/mmol) and [α-^{32}P] rUTP (1500 Ci/mmol) were all purchased from Bresatec. [^{3}H] -Leucine was obtained from Amersham as was ^{125}I-iodide.

6.2.4 Buffers

Denhardt's solution : 0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidine, 0.1%(w/v) BSA

NET : 100mM NaCl, 1mM EDTA, 10mM Tris HCl pH 7.5

SSC : 150mM NaCl, 15mM sodium citrate

SSPE : 150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA

TAE : 40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, pH 8.2

TBE : 90mM Tris, 90mM boric acid, 2.5mM EDTA, pH 8.3

TE : 10mM Tris-HCl pH 7.5, 0.1mM EDTA

TES : 25mM Tris-HCl pH 8.0, 10mM EDTA, 15% sucrose

TEG: 25 mM Tris-HCl pH 8.0, 10mM EDTA, 20 % glucose

HEPES buffered saline: 20 mM HEPES, 137 mM NaCl and 5 mM potassium chloride, pH 7.05 (Hepes buffered saline with 6 mM glucose was used as the H4IIE electroporation buffer)

All buffers were sterilized by autoclaving or where necessary by filtration through a Sartorius ™ Minisart NML 0.2μm filter.

6.2.5 Bacterial Strains and Bacterial Growth Media

i) Bacterial Strains

The following *E.coli* K12 strains were used :

(1) *E. coli* BB4 : *sup* F58 *sup* E44 *hsd* R514 *gal* K2 *gal* T22 *trp* R55 *met* B1*ton* A ΔlacU169 F'[proAB$^+$ lacI^q lacZΔM15 Tn10 (*tet* ^{r})] host for recombinant plasmids and M13 bacteriophage.

(2) *E.coli* DH5a: *sup* 44 Δ*lac* U169 (p80 *lac* ZΔM15) *hsd* R17 *rec* A1 *end* A1 *gyr* A96 *thi*-1 relA1 host for recombinant plasmids, obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven.

(3) *E.coli* XL1-Blue : *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ F'[*pro*AB$^+$ *lac*I^q lacZΔM15 Tn10 (tet^r)] host for recombinant plasmids and M13 bacteriophage, purchased from Stratagene.

(4) *E.coli* JM101: Δ (*lac-pro*), F' *Lac* I^q Z Δ M15, *trad* I host for recombinant plasmids purchased from Stratagene

(5) *E.coli* CJ236: *dut, ung, thi, rel* A; pCJ105 (*Cm*^{r}) host for plasmids undergoing site-directed mutagenesis.

(6) *E.coli* MV1190: Δ (*lac-pro* AB), *thi*, *sup* E, Δ (*sri-rec* A)306:: Tn10 (*tet* ^{r}) [F': *tra* D36, *pro* AB, *lac* [^{q} Z Δ M15]

Stock cultures of these (and plasmid transformed bacteria) were prepared by dilution of an overnight culture with an equal volume of 80% glycerol and stored at either -20 ^{o}C, or -80 ^{o}C for long term storage. Single colonies of bacteria, obtained by streaking the glycerol stock onto agar plates of suitable medium (Section 2.1.9) were used to innoculate liquid growth medium, and the bacterial cultures were grown at 37 ^{o}C with continuous shaking to provide adequate aeration.

ii) Bacterial Media

Growth media were prepared in double-distilled water and sterilized by autoclaving, antibiotics and other labile chemicals were added after the solution had cooled to 50 °C.

(1) Luria (L) broth : contained 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH. Agar plates were prepared by adding 1.5% (w/v) Bacto-agar (Difco) to the L broth. Ampicillin (50 μg/ml) or tetracycline (10 μg/ml) or chloramphenicol 170 μg/ml) were added where appropriate for growth of transformed bacteria, to maintain selective pressure for the plasmid.

(2) 2 x YT Medium : 1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl, adjusted to pH 7.5 with NaOH.

(3) Solid Media : Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar.

6.2.6 Cloning Vectors

pBS KS^- and pBS SK^+ were purchased from Stratagene.

pGEM 3Zf(+) was purchased from Promega.

pSP72 and pSP73 were purchased from Bresatec

6.2.7 Cloned DNA sequences

The following cloned DNA sequences used as probes throughout this study were generous gifts from the following :

pMTIIa-pGH: Dr. J.R.E. Wells, Biochemistry Dept., University of Adelaide

pHFb-Actin : Dr. L. Matchoss, Stanford University, California.

pSV40hGH : Mr. G. Ryan, Institute of Medical and Veterinary Science. Adelaide.

pRr18S cDNA for 18S ribosomal RNA : Dr J Mercer, Murdoch Institute, Melbourne.

pPGH sp72 :Mrs. L. Crocker, Biochemistry Dept., University of Adelaide

pSP72 *Neo* ^{R} : Mr. Tim Sadlon, Biochemistry Dept., University of Adelaide

pGEM rIGF-I and pGEM rBP-1 : Dr. L. Murphy, Dept. of Physiology, University of Manitoba, Winnipeg, Manitoba R3E OW3, Canada

pGEM rGHR/BP: Dr. A.C. Herington ,Royal Childrens Hospital Melbourne Vic.

pBH1.2 and pPCK10 : Professor R.W.Hanson, Biochemistry Dept., Case Western Reserve University ,Cleveland,Ohio)

6.2.8 Oligonucleotides:

All oligonucleotides were synthesised by Bresatec Ltd.

MTIIa-pGH chapter:

1367 (26 mer): 5'-ATG AGA ATA CTG GAG AGT CCT TAT CT-3'

1384 (26 mer): 5'-ATG ACG TAT CCG AAG CTA GTT GAG TA-3'

both primers bind within the hMTIIa promoter region (Karin and Richards 1984) and amplify a 130 bp region

Cell Culture chapter:

17 mer sequencing primer: 5'-GCC ATA CCC TGT GGG CT-3' (binds to the codons for lysine 27 of mature hIGF-I to glycine 32)

RRS 644: (30 mer) 5'-AGC CCC GCA GAG CGT*AGC CGT GGC AGA GCT-3' (removes the three N-terminal amino acids of hIGF-I to produce des(1-3)IGF-I.

#1655: (30 mer) 5'-GGT CTG GAT CAG **AAG CTT** TGC GGT GGC ATG-3' (creates a Hind III site at 3'end junction of hIGF-I cDNA and PEPCK exon 1, to enable removal of PEPCK intron / exon sequences)

PEPCK-IGF-I Transgenesis chapter:

#2024 (18 mer): 5'-GTT CAA TCA TTA TCT CCC-3' (binds in PEPCK promoter region)

#2025 (20 mer): 5'-GGT AGA AGA GAT GCG AGG AG-3' (binds in hIGF-I cDNA)

6.2.9 Tissue Culture Cell lines and Media

Rat Reuber H4IIE hepatoma cells and Chinese Hampster Ovary (CHO) cell lines were purchased from the American Type Cell Culture (ATCC) Laboratory. Rat L6 myoblasts were provided by Dr.J.M.Gunn, Dept. of Biochemistry and Biophysics, Texas A and M University College Station, Texas, USA.

Phosphate-buffered saline (PBS) : 136mmol /L NaCl, 2.6mmol/L KCl, 1.5mmol/L KH_2PO_4 and 8 mmol/L Na_2HPO_4, pH 7.4, was sterilized by autoclaving (20 psi for 25 min at 140 ^oC).

Trypsin/EDTA solution : 0.1% trypsin (Difco) and 1 x EDTA Versene buffer solution (CSL), was sterilized by filtration through a 0.2μM filter (Whatman).

Growth Medium for H4IIE and L6 cells: 1 x 1 litre packet Dulbecco's minimal essential medium, (DMEM) (Gibco), 28mmol/l $NaHCO_3$, 19mmol/l glucose, and 20mmol/l

Hepes, pH 7.3, was supplemented with 50,000 Units Gentamycin (Gibco), and filter sterilized as described above.

Growth medium for CHO cells : 1 x 1 litre packet Ham's F12 with L-glutamine (Gibco), and 28mmol $NaHCO_3$, was supplemented with 50,000 Units Gentamycin (Gibco), pH 7.4, and filter sterilized as described above.

Fetal Calf Serum : Commonwealth Serum Laboratories, Melbourne , Australia

Fetal Bovine Serum: Flow Laboratories , North Ryde, N.S.W., Australia

6.2.10 Proteins and Growth Factors

Molecular Weight Protein Standards: Biorad, N.S.W., Australia

hIGF-I : Gropep Ltd, Adelaide, S.A., Australia

pGH: Bresatec Ltd., Adelaide, S.A., Australia

anti-pGH Antibody: UCB, Bioproducts

anti-hIGF-I polyclonal Antibody: Dr. P.C.Owens,CSIRO Division of Human Nutrition, Adelaide, South Australia

anti-hIGF-I monoclonal Antibody: Dr. R. Baxter, Royal Prince Alfred Hospital, Camperdown, N.S.W, Australia

6.2.11 Miscellaneous items

GF/A glass fibre filter discs and 3MM paper : Whatman Ltd

Kodak Diagnostic film X-Omat AR, USA

Nitrocellulose (BA 85) and Nytran 0.45µm : Schleicher and Schuell

X-ray film : Fuji Photo Film Co. Ltd, Tokyo, Japan

6.3 METHODS:

6.3.1

i) Containment facilities

All manipulations involving recombinant DNA technology and transgenic animals were carried out under C1 containment conditions in accordance with the regulations and approval of the Genetic Manipulation Advisory Committee (GMAC) and the Adelaide University council Experimental manipulation of animals was carried out with the permission of the animal Ethics Committee at the University of Adelaide.

ii) General DNA methods

The following methods were performed essentially as described in Maniatis *et al.*, (1984) :

Growth, maintenance and preservation of bacterial and viral strains; quantitation of DNA and RNA; autoradiography; agarose and polyacrylamide gel electrophoresis; DNA and RNA precipitations; phenol/chloroform extractions; end-filling or end labelling of DNA fragments using the Klenow fragment of *E.coli* DNA polymerase I.

6.3.2 Plasmid DNA preparation

The rapid alkaline hydrolysis procedure of Birnboim and Doly (1979) was used for the isolation of plasmid DNA from 2.5 ml overnight cultures for analytical restriction digests. This method was also employed for the bulk preparation of plasmid cDNAs from 50 ml cultures for use as probes either for radiolabelling in Dot-blot, Southern or Northern hybridization analysis.

DNA used for transfection of tissue culture cell lines or for double stranded DNA sequencing, was routinely grown up in 100ml cultures innoculated with 1ml from a 5ml overnight culture. The plasmid was extracted using the alkaline lysis procedure described above and further purified by gel filtration on the HPLC following treatment with RNase A (2 mg/ml), using a Superose-6, preparative grade column matrix (Pharmacia) to remove any contaminating RNA, thereby allowing accurate quantitation of the DNA by spectrophotometry.

6.3.3 Isolation of Genomic DNA

i) from animal tails: Tail samples (about 1 cm) were obtained from mice or rats at approximately four weeks of age (at the time of weaning) snap frozen in liquid Nitrogen and stored frozen until required. Tails were sliced into small sections and resuspended in a solution (1 ml) containing 1 % (w/v) SDS, 50 mM Tris-HCL pH 7.5, 10 mM EDTA and 50 µg/ml proteinase K. The samples were then digested overnight at 37 ^{o}C before being spun briefly at 2000 rpm to pellet any cell debri. The supernatant was then extracted twice with phenol/chloroform and once with chloroform before being digested with RNAse A (50 µg/ml) for 60 minutes at 37 ^{o}C. Subsequently, the samples were subjected to a single phenol/chloroform extraction followed by an additional chloroform only extraction.

The DNA was then collected by ethanol precipitation and its concentration determined by measuring the UV absorbance at a wavelength of 260 nm. The concentration, integrity and contamination with RNA was then checked by running an aliquot of each preparation on an agarose mini-gel.

ii) from tissue-culture cells: In most cases one confluent 150 cm^3 flask of H4IIE or CHO cells was washed with two changes of sterile PBS and the cell monolayer trypsinised. After washing the cell pellet in PBS it was resuspended in 0.5-1.0 ml of digestion buffer and digested overnight at 37 ^oC Essentially the same protocol as above was then used for extraction of DNA from the cell pellet.

6.3.4 Restriction enzyme digestion and analysis of DNA

In analytical digests, 0.5-1 µg of DNA was incubated with 2-5 units each of the appropriate restriction enzyme(s) for a minimum of 2 hours in the buffer conditions specified by the manufacturer or in 1 x "super dooper" buffer.(30 mM Tris HCl pH 7.8, 625 mM potassium acetate, 100mM Magnesium acetate, 40 mM spermidine and 5mM DTE) Reactions were terminated by the addition of a 1/3 volume of urea load buffer and electrophoresed on 1% or 0.8 % mini-agarose gels in TBE buffer.

In preparative digests, 5-20 µg of DNA was restricted in a reaction volume of 50 µl-100 µl using a two to four-fold excess of enzyme, and the desired DNA fragments were isolated as detailed below.

Genomic DNA was routinely precipitated with spermine before restriction digestion to facilitate digestion. Samples in TE buffer (usually 10-50 µg of DNA) were precipitated for 15 minutes on ice with 2 mM spermine. After spinning at 10,000 rpm for 15 minutes the pellet was resuspended in 100 µl of displacement buffer containing 0.3 M Sodium acetate pH 5 and 10 mM $MgCl_2$. The DNA was then collected by ethanol precipitation and digested overnight in 1 x "super dooper" buffer, using a four-fold excess of enzyme.

6.3.5 Preparation of restriction fragments and vectors

All restriction fragments for use in ligation or as probes were isolated from either a horizontal 0.8%-2.0% agarose gel, depending on the size of the DNA restriction fragment(s). Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragment(s) excised from the

gel. The DNA containing gel slice was then placed in dialysis tubing and DNA electroeluted into 200-500 µl of TE buffer at 60 mA for 30 min. Immediately before stopping the elution the direction of current flow was briefly reversed to remove any DNA adhering to the tubing. The eluent was then extracted with phenol/chloroform and chloroform singly before the DNA was recovered by ethanol precipitation. Alternatively digests were run on 0.8%-2.0% TAE agarose gels and the DNA isolated using the Geneclean™ protocol.

The Geneclean protocol was used exclusively in preparation of transgene DNA for microinjection. In these cases the final elution of the DNA was carried out into sterile PBS.

Plasmid vectors for cloning (pBH1.2, pBS, pSP72/73) were linearized with the appropriate restriction enzyme(s) by digestion for 2-4 hours only. In cases where complementary termini were produced, to prevent self-ligation of the vector, 5' terminal phosphate groups were removed by incubation in 50mM Tris-HCl pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, with 0.5 units of calf intestinal phosphatase (CIP), in a final volume of 50 µl for 1 hr at 37 °C The vector DNA was isolated after electrophoresis on a 1.0% agarose TAE gel using a Geneclean™ kit according to the manufacturers' instructions. The DNA was resuspended at a concentration of 20-50 ng/µl, for use in ligation reactions.

6.3.6 Ligation of DNA

A 20µl reaction contained 20-50ng of vector DNA, the DNA restriction fragment, 50mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 1mM DTT, 1mM ATP, and 1-2 units of T4 DNA ligase. For cloning into plasmid vectors, a 2-3 molar excess of restriction fragment insert to vector DNA was used. The reactions were incubated for either 4 hours or overnight at 14 °C. A control ligation with vector only was set up and included in the subsequent transformation to determine background levels of uncut or recircularized vector DNA. In some cases a ligation containing insert only was also carried out to assess the contamination of insert stocks with un-cut DNA.

6.3.7 Transformation

A single colony of the *E. coli* host strain was innoculated into 5ml of L-broth (where appropriate the L-broth was supplemented with an antibiotic) and the culture incubated overnight at 37 °C with continuous shaking. The overnight culture was then diluted 100 fold into 50ml of L-broth (plus antibiotic) and the incubation continued at 37 °C, with shaking, until the culture reached an absorbance at A_{600} of 0.6-0.8. The cells were

then pelleted by centrifugation at 2,000 x g for 5 min, resuspended in 2.5ml of ice cold 0.2M $MgCl_2$, 0.5M $CaCl_2$ and left on ice for 60 min. Two hundred microlitres of this cell suspension was mixed with 2-5µl of the DNA ligation reaction mix (Section 2.2.6) and left on ice for 40 min. The cells were then heat shocked at 42 °C for 2 min, L-broth plus 20mM glucose was added (100µl if the cells were spread directly onto the agar plates), and the cells were incubated at 37 °C for at 20-30 min. The transformed cells were then plated onto L-agar containing 50µg/ml of ampicillin [+/- BCIG (50 mg/ml) and IPTG (20 mg/ml)], by spreading with a wire spreader. The agar plates were routinely incubated at 37 °C overnight.

6.3.8 Preparation of single-stranded DNA

Single-stranded DNA was prepared for both sequencing and site-directed mutagenesis as follows. Single colonies of transformed bacteria were inoculated into 1.5ml cultures of L broth containing 0.001% thiamine and 50µg/ml ampicillin, and grown overnight at 37 °C with good aeration. The cultures were then diluted 1:40 grown to an A_{600} of 0.6-0.8, and again diluted 1:40. M13K107 helper phage at a multiplicity of infection of 10 was added, and the cells were incubated at 37 °C for 1 hour. Kanamycin (70µg/ml) was then added and the cells incubated overnight at 37 °C with good aeration.

Cells were pelleted by centrifugation in an Eppendorf centrifuge for 5 min. The supernatant was carefully removed into a fresh tube and recentrifuged. Two hundred microlitres of 2.5M NaCl , 20% PEG 6000, was added to 1.2ml of the supernatant. After 15 min at room temperature the single-stranded phage pellet was collected by centrifugation in an Eppendorf centrifuge for 5 min. All traces of the supernatant were removed, and the pellet was resuspended in 100µl of 10mM Tris-HCl pH 8.0, 0.1mM EDTA and extracted with an equal volume of buffer-saturated phenol. The aqueous phase was re-extracted 3 x with 500µl of diethyl ether and ethanol precipitated. The phage DNA was collected by centrifugation, washed in 70% ethanol, air dried, resuspended in 25µl TE buffer, and stored at -20 °C. In some cases the DNA was treated with RNase A (50 µg/ml), phenol/chloroform extracted and re-ethanol precipitated prior to storage or use.

6.3.9 Di-deoxy chain termination DNA sequencing

Single-stranded template DNA (approximately 500 ng) was annealed in 10mM Tris-HCl pH 8.0, 1mM $MgCl_2$ with 5-7 ng of the appropriate primer (1µl) in a final volume

of 10µl. The mixture was heated at 95 °C for 3 minutes, and incubated at 50 °C for at least 15 minutes.

Sequencing was performed using the dideoxy method (Sanger et al., 1977), with the sequencing reagents supplied in the Bresatec DSK-A kits. The sequencing reactions were performed in accordance with the protocol accompanying the kits.

Double-stranded sequencing was performed using plasmid DNA (3-5 µg per reaction) purified on a Superose-6 gel filtration column (Pharmacia). The DNA was denatured in 0.2M NaOH, 2mM EDTA for 30 min at 37 °C. The mixture was then neutralized by the addition of 0.1 volumes of 3M sodium acetate pH 4.6 and the DNA precipitated with 3 volumes of ethanol. The DNA pellet was collected by centrifugation, washed in 70 % ethanol, and resuspended in 7µl of 0.1mM EDTA prior to sequencing. The sequencing reactions were carried out as for single-stranded DNA, using either the DSK-A kit (Bresatec) or a Sequenase® version 2.0 kit in accordance with the protocol accompanying the kit.

Aliquots of each sequencing reaction (1-2 µl) were analysed on 6 % acrylamide/8 M urea gels run in 1 x TBE buffer. Gels were pre-electrophoresed at 20 mA before use and were run at 30 mA constant current. Following electrophoresis gels were fixed for 10 minutes with 10 % (v/v) acetic acid and subsequently washed with 20 % (v/v) ethanol for 10 minutes prior to being baked dry. Dried gels were then exposed to X-ray film at room temperature for 4-24 hours.

6.3.10 Preparation of [^{32}-P]-Labelled DNA probes (Oligo-labelling of DNA)

In all experiments, a Bresatec Hexaprime kit was used for the oligolabelling of recombinant plasmids. 0.1-0.5 µg of DNA was labelled with ^{32}P in a 20µl reaction containing 100mM Tris-HCL pH 7.6, 20mM $MgCl_2$, 100mM NaCl, 200µg/ml BSA, 4µM each of unlabelled dGTP and dTTP, 100µCi each of [α-^{32}P] dATP and [α -^{32}P] dCTP and 5.0 units of the large Klenow fragment of *E coli* DNA polymerase I.

The reaction mix was incubated at 37 °C for 30 minutes and then extracted with phenol/chloroform . The probe was then precipitated with ethanol (3 volumes) in the presence of 0.75 M ammonium acetate pH 5.5 buffer and 2 µl of glycogen (Boehringer Mannheim) at -80 °C for 15 minutes The DNA was pelleted by centrifugation for 30

minutes at 12,000 x g, washed with 1 ml of ice-cold 70% ethanol, air dried and resuspended in 200µl of TE buffer.

The specific activity of probes was generally 1-2 x 10^8cpm/µg. Immediately before adding to the hybridization mix, the the oligo-labelled DNA was denatured by incubation at 100 °C for 10 minutes, after the addition of 10M NaOH to a final concentration of 0.3M. The probe was then snap cooled on ice and neutralised by the addition of an equal volume of 4M ammonium acetate.

6.3.11 Kinasing of oligonucleotides

Briefly, stocks of crude oligonucleotides (50µg) varying between 75-98 % in purity were ethanol precipitated with 0.3 M sodium acetate pH 5.5 and 3 volumes of ethanol before being resuspended in 20 µl TE buffer. Oligonucleotides were then run on a 20% polyacrylamide gel at 32 mA for 60 minutes and the major, highest molecular weight species excised from the gel after visualising the DNA bands by overlaying the gel on a TLC plate under UV light. The oligonucleotide was then eluted overnight in water, quantitated, ethanol precipitated and resuspended in TE buffer at a desirable concentration .

The purified synthetic DNA oligonucleotides used in mutagenesis were kinased prior to use. The reaction mixture contained 10mM $MgCl_2$, 50mM Tris-HCl pH 7.4, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 1 mM ATP and 2 units of T4 polynucleotide kinase in a final volume of 10µl. This was incubated at 37 °C for 30 minutes and the reaction stopped by freezing at -20 °C.

6.3.12 Site-directed mutagenesis

Site-directed mutagenesis was carried out using a Bio-rad Muta-gene™ kit according to the manufacturers instructions. The template to be mutagenised was transformed into competent *E.coli* CJ236 cells (these cells were plated on L-broth + chloramphenicol (30 µg/ml) agar plates) as in 6.3.7 and single-stranded uracil containing DNA prepared as in 6.3.8.

Approximately 0.1pmol of single-stranded uracil containing DNA was annealed separately with several different concentrations of 5' phosphorylated mutagenesis primer (2-5 pmol) in reactions containing 20 mM Tris-HCl (pH 7.4), 2mM $MgCl_2$ and 50 mM NaCl and water to a final volume of 10 µl. An additional reaction tube in which no primer was added, was set up to test for non-specific priming. The mixture was heated to 70 °C for 5

min and left to cool at room temperature for a further 40 minutes to allow annealing of the mutagenic primer.

Synthesis of the second strand was carried out in each annealing reaction with 0.4 mM of each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl (pH 7.4), 3.75 mM $MgCl_2$ and 21.5 mM DTT final concentration. To this mixture 1 unit of T4 DNA polymerase and 2-5 units of T4 DNA ligase were added. The reactions were incubated on ice for 5 minutes, at 25 °C for 5 minutes and finally at 37 °C for 90 minutes before the reaction was stopped by freezing at -20 °C. To ensure second strand synthesis had occurred, aliquots of each reaction (5 µl) were electrophoresed on 1 % agarose gels and the mobility of the single-stranded template DNA compared with the mutagenic reactions.

The remainder of each mutagenesis reaction was then transformed into competent MV1190 cells. Plating of the transformed cells on L-broth + Ampicillin plates usually led to the recovery of 20-100 colonies and no colonies on the reactions carried out in the absence of primer. An extremely high rate of mutagenesis was achieved using the kit as above (successful mutagenesis was achieved in 90 % of clones picked). Mutations were confirmed by sequencing and/or restriction analysis of individual clones.

6.3.13 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) was carried out in the identification of transgenic animals and was performed using a Bresatec Taq polymerase kit according to instructions. A master mix was first produced containing **n**x(5 µl)10 x Taq reaction buffer, **n**x(4 µl) 25mM $MgCl_2$, **n**x(0.5 µl) 20 mM dNTP (all four),**n**x primer 1, primer 2 (between 300-500 µg/ml) and **n**x(1ul) 0.1 U/ul Taq polymerase where **n** is the number of reactions to be carried out. Aliquots (14 µl) of the master mix were then removed to each reaction tube and the volume in each tube made up to 48 µl with sterile water. Finally, template DNA (2 µl) was added to each reaction tube and the reactions sealed with the addition of sterile paraffin oil. PCR was performed with a Perkin Elmer Cetus Thermal Cycler (Norwalk, CT, USA). Each PCR run included a contamination control lacking template DNA and a tube in which a negative control DNA template was used. All manipulations above were carried out using dedicated positive displacement pipettes in a laminar flow hood to prevent contamination. The reaction conditions used for individual PCR reactions are described in the relevant results chapters.

Following amplification, aliquots (5 µl) of the 50 µl reactions were analysed by electrophoresis on 2 % agarose gels run in TBE buffer. The reaction products were visualised by illumination under UV light.

6.3.14 DNA Dot-blot

DNA (5ug) extracted from the tails of rats or mice was denatured in 0.1M NaOH, 2M NaCl and applied to Schleicher and Schluell Nytran™ membrane using a Millipore Dot-blot manifold apparatus. Samples were washed through with 2M NaCl and the DNA crosslinked to the membrane using a Stratagene UV Crosslinker. The filter was then prehybridised overnight at 42 °C in a solution containing 50 % Formamide, 10x Denhardts soln., 1% SDS, 1M NaCl, 50mM Tris-HCl pH 7.5, 10% Dextran Sulphate and 100 µg/ml sonicated/denatured salmon sperm DNA. Blots were hybridised in the same solution with a heat denatured ^{32}P-labelled probe. All probes were generated using a Bresatec Hexaprime labelling kit. Filters were typically washed after overnight hybridisation, at room temperature in a solution containing 2XSSC, 0.1% SDS (30 min.) followed by a wash at 42 °C (30 min). Finally the filters were washed at room temp and 42 °C or 65 °C (30 min) in a solution containing 0.1xSSC, 0.1% SDS, before autoradiography at -80 °C.

6.3.15 Southern analysis and hybridisation conditions

DNA was digested with the appropriate restriction enzymes and electrophoresed on a 0.8-2.0% (depending on the size of the DNA fragments being separated) agarose gel in 1 x TBE buffer. Following staining with ethidium bromide, the gel was visualised under UV light, and photographed. The DNA fragments were partially hydrolyzed by soaking the gel in 0.25M HCl for 20 minutes, denatured, by soaking the gel in 0.5 N NaOH/1M Tris pH 7.5 for 20 minutes and neutralised in 1.5M Tris pH 8.0 before transfer to Nytran™ according to the method of Southern, as described by Maniatis *et al.*, (1984). Following transfer, the filters were either baked *in vacuo* for 2 hrs, or the DNA was cross-linked to the nylon membrane using UV light in a Stratagene Strata-linker 1800 for 3 minutes.

Prehybridization was then performed for 4-24 hours in 6 x SSC, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidine, 0.1% (w/v) BSA, 0.1% SDS, 50 % deionised formamide, 10 % dextran sulphate and 200 µg/ml of heat-denatured sonicated salmon sperm DNA, at 42 °C. Hybridization was carried out for 24-48 hours in the same solution at 42 °C with the addition of 20-50 ng/ml of [^{32}P]-oligo-labelled DNA probe. Filters were washed in 1 x SSC, 0.1% SDS for 15 minutes at room temperature, followed by one wash at 42 °C in the same buffer. Subsequently, the filters were washed at room temperature and then 42 °C in 0.1 x SSC, 0.1% SDS before autoradiography . In some cases a further wash of 30-60 minutes in 0.1 x SSC, 0.1% SDS at 65 °C was performed. Autoradiography of the filter was performed overnight or longer at -80 °C, depending on the strength of the hybridization signal.

6.3.16 Isolation of RNA

Total RNA was extracted from animal tissue (0.5 -1.5 g) using the guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (1987), modified in the following way. Firstly, the volumes used in the published extraction procedure were scaled up. Secondly when RNA is prepared from liver, glycogen tends to co-purify with the RNA, this was removed by precipatation of the RNA with 3 volumes of 4M Na acetate at 0 °C overnight. In some cases as a final purification step instead of Na acetate precipitation, the RNA was precipitated overnight at 0 °C with five volumes of 4M LiCl. The RNA was recovered by centrifugation at 8000 x g for 15 min at 4 °C, and resuspended in 0.1mM EDTA.

Total RNA was extracted from tissue culture cells, essentially as described by Chomzynski and Sacchi (1987). Cells were first trypsinised from 75 cm^3 or 150 cm^3 flasks and the cell pellet washed three times in PBS prior to extraction with solution D (4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol)

In both cases, following RNA extraction, the absorbance values at 260 nm and 280 nm of each RNA sample were determined on a Shimadzu UV-160A spectrophotometer. The A260/A280 ratios of the RNA samples were consistently in the range 1.6 - 2.0. The relationship of one A260 unit equal to 40 µg/ml RNA was used in the calculation of RNA concentrations. Aliquots of RNA were analysed on 1% Agarose TBE gels to assess the integrity of the 18 and 28S ribosomal RNA species prior to use.

6.3.17 Analysis of RNA

i) Northern analysis

Northern hybridization analysis of total RNA was carried out by denaturation on 1% agarose gels containing 1.1M formaldehyde, 0.02 M Sodium Phosphate buffer pH 7.4, and transfer onto Nytran™ (Schliecher and Schuell) filters. Prior to transfer gels were washed for 30 minutes in 10 x SSPE to remove formaldehyde. Transfer was carried out overnight in either 10 x SSPE or HETS™ (High Efficiency Transfer Solution) Following transfer, the filters were either baked for 2 hours *in vacuo*, or irradiated with 120mjoules of UV radiation for 3 minutes in a Stratagene UV Stratalinker™ 1800 which results in the RNA being covalently crosslinked to the filter (manufacturer's instruction manual). Filters were pre-hybridized for 4-16 hours at 42 °C in 50% formamide, 5 x SSC, 5 x Denhardt's solution 0.1% SDS, 0.05% sodium pyrophosphate, and 200 µg/ml of sonicated salmon sperm DNA. Hybridizations were carried out for 18-24 h under exactly the same conditions, except for the addition of radiolabelled probe (1-5 x 10^8 counts/µg). In the case of riboprobes, prehybridisation and hybridisation were at 60 °C. Filters were usually washed in 2 x SSC, 0.1% SDS at room temperature for five and twenty minutes, followed by one wash in 2 x SSC, 0.1% SDS at 60 °C or 65 °C for 40 minutes.

ii) *In vitro* synthesis of [^{32}P] labelled RNA

Antisense RNA probes were prepared using a Bresatec *in vitro* RNA transcription kit according to the manufacturers instructions. Ten micrograms of the DNA vector containing the anti-sense DNA template for RNA synthesis was linearized with the appropriate enzyme, separated from any uncut vector by electrophoresis and the template recovered by the Geneclean™ protocol. Alternatively, following complete digestion with the appropriate restriction enzyme the template DNA was purified by gel filtration on a Sepharose-6 column (Pharmacia). One microgram of this DNA was added to a reaction mix containing 0.01M DTT, 0.04M.Tris-HCl pH 7.6, $MgCl_2$, 500µM of each ATP, CTP and GTP, 50µM UTP, 1µg BSA, 100µCi [One microgram [α-^{32}P] UTP and 4 units of T7 RNA polymerase, in a total volume of 20µl. The reaction was incubated at 37 °C for 60 minutes, before the addition of RNase-free DNase I (5-10U) and further incubation at this temperature for 20 minutes. The reaction was then phenol/chloroform extracted and the probe precipitated in the presence of 0.3M Na acetate pH 5.5, glycogen (1ug) and 3 volumes

of ethanol at -80 °C for 15 minutes. The probe was then collected by centrifugation at 10,000 x g for 15 minutes and electrophoresed on a 6% polyacrylamide gel. The gel was then autoradiographed for 2-3 minutes and the band corresponding to the full length probe was excised. The labelled probe was eluted overnight in 400µl of elution buffer (0.5M ammonium acetate, 1mM EDTA,and 0.1% SDS), at 37 °C. The buffer containing the probe was then aspirated away from the gel slice and the RNA was stored at -20 °C.

iii) Ribonuclease protection analysis

This assay was carried out using the Ambion RPA II™ kit supplied by Bresactec Ltd., in accordance with the protocol provided. Test RNA (20 -50µg) and approximately 50,000 cpm of the single-stranded RNA probe were combined in an Eppendorf tube and pelleted by centrifugation following ethanol precipitation. The supernatant was removed and the RNA pellet dissolved in 20µl of deionized formamide, 3µl of water and 3µl of 10 x hybridization buffer (4M NaCl, 0.4M PIPES pH 6.4, and 0.01M EDTA). The mixture was heated to 85 °C for several minutes and then incubated at 55 °C overnight.

Following hybridization, 200µl of RNase digestion solution was added (0.3M NaCl, 0.01M Tris-HCl pH 7.5, 0.005M EDTA) with a mixture of RNase A (50 units/ml) and RNase T1 (10,000 units/ml) diluted 1/100. Each assay included a tube in which digestion buffer was added without enzyme. The reaction was incubated at 37 °C for 30 min.before 300 µl of RNase inactivation/precipitation mixture was added and the tubes kept at -20 °C for 15 minutes before being centrifuged at 10,000 rpm for 15 minutes to precipitate the hybrids. Alternatively, 225 µl of Solution D (see Chomczynski and Sacchi (1987)) was added, the tubes vortexed briefly and 5 µl of tRNA (1 µg/ul) added before the mixture was precipitated with an equal volume (approximately 450 µl) of isopropanol. The protected RNA was dissolved in formamide loading buffer (Maniatis *et al.*, 1982), denatured by heating at 85 °C for 2 min and analyzed by electrophoresis on a 6-8% sequencing gel. The gels were autoradiographed at -80 °C for approximately 2-7 days using Kodak X-Omat sensitive film.

6.3.18 Creation of permanent tissue culture cell-lines

i) Cell maintenance

All cells were routinely maintained in 150 cm^3 flasks at 37 °C in an atmosphere of 5% CO_2 and were subcultured every 3-4 days. To subculture or harvest the cells the culture media was removed and the cells washed in PBS before the addition of 3mls of

trypsin/EDTA solution. The cells were left at room temperature until they began to detach from the flask, when 7mls of culture media was added and the flask washed to remove any remaining cells. The cells were subcultured by routinely being split 1:4, into fresh media and incubating at 37 °C. If the cells were to be harvested they were washed twice in 10mls of PBS, and pelleted by centrifugation at 1200 x g for 5 minutes, before resuspending in the appropriate buffer.

Transfection of rat Reuber H4IIE hepatoma cells by electroporation

Transfection of rat H4IIE cells by electroporation was performed by a modification of the method of Chu *et al.*, (1987) and was also based on the procedure used for electroporation of rat H4IIE C3 cells by Dr. G. Bradiotti (personal communication). To ensure the cells were in the growth phase they were routinely split 1:2 the day before electroporation

The cells were detached using trypsin/EDTA, washed 2 x in PBS and resuspended in Hepes buffered saline (HBS) containing 0.02M Hepes pH 7.05, 0.137M NaCl, 0.005M KCl, 700μM $Na_2 HPO_4$, 0.06M glucose at a concentration of 1x 10^7 cells/ml. Sonicated salmon sperm (250μg), 15μg of the DNA construct to be transfected and 500μl of the cell suspension was added to each electroporation cuvette and gently mixed using a pipette. Cuvettes were also set up in which there was no DNA for transfection.

The cells were incubated at 4 °C for 10 min, exposed to a single voltage pulse of 240 Volts, Capacitance 960, and left at room temperature for 10 min before gently plating into 60mm dishes containing 4mls of DMEM plus 10% FCS serum. Cells were incubated at 37 °C for 48-72 hours. Following this period the media was changed to DMEM + 10 % FCS plus the Neomycin analogoue, G418 at 500 μg/ml. The selective media was changed every two days until approximately one week after commencement of selection, at which stage all the cells from the no DNA, mock electroporation had been killed off. At this stage individual G418-resistant colonies had begun to form on plates of cells electroporated with DNA constructs. When colonies were composed of 50-100 cells the plates were washed with PBS and the individual colonies trypsinised away from each other and transferred to 25 cm^3 flasks where selection was maintained. From 25 cm^3 flasks each clone picked was expandend up until there were enough cells to freeze down (usually 2 x 75 cm^3flasks).

Cells for freezing down were trypsinised, pooled and spun down at 1200 x g before being resuspended in 1 ml of FCS +10 % DMSO and immediately transferred to -80 °C.

iii) Transfection of chinese hamster ovary cells (CHO) by electroporation

Mycoplasma-free CHO cells were harvested and transfected by electroporation as described above, using the following conditions. The cells were resuspended in PBS at a concentration of 6.25 x 10^6 cells per ml, and 800µl of the cell suspension was used for each transfection. The electrical pulse was 1800 volts and 240 capacitance, and the cells were plated into 4mls of F12 media, and treated as described above. Selection of permanent cell lines was carried out as for H4IIE cells except for the lower concentration of G418 used in selection (450 µg/ml). Furthermore, CHO cells were maintained in F12 medium with the addition of 10 % FCS as opposed to DMEM .

6.3.19 L6 Protein Synthesis assay

This assay was carried out as described by Ballard et al., (1986) using monolayers of rat L6 myoblasts grown to confluence in 24 place multiwell dishes (Gibco). The serum containing DMEM growth medium was removed from from each plate and each well washed twice with 1 ml of WMEM (a 1:1 mixture of Waymouths Essential Eagles Medium and DMEM) for one hour each wash. This serum-free medium was then removed and each well was set up in 0.90 ml of the following labelling mixture: WMEM containing 100 mM leucine and ^{3}H-leucine (47MCi/mmol). The appropriate sample or treatment was added to the wells in a volume of 0.1ml and the plates were incubated overnight at 37 ^{o}C in a humidified incubator with 5 % C02 (v/v) in air. Following the overnight incubation the plates were washed with 2 x1ml of Hanks Balanced Salts, 2 x 1ml 0.5 % trichloroacetic acid (each wash 5 minutes) and 1 x 2ml of glass distilled water. The monolayer was then digested with 1 ml/well of 0.5 N NaOH/0.1 % Triton X-100 for 10 minutes before 2 x 0.2 ml was subsampled from each well for ß counting with scintillant. Each assay included a negative control in which 0.1 ml of sterile PBS replaced the treatment for each well. The positive controls were 10 % FBS and increasing concentrations of hIGF-I. Samples were generally assayed in triplicate although in some cases duplicates were assayed when large numbers of samples were to be assayed in the one experiment.

6.3.20 Gel filtration of plasma samples and culture supernatants

Prior to assay of plasma samples from transgenic animals and culture supernatants for IGF-I it was necessary to remove IGFBPs from the samples. IGFBP can interfere in the radioimmunoassay used for IGF-I detection. The most rigorous way of removing IGFBP from IGF is to carry out gel filtration chromatography of samples under acid conditions (Owens et al., 1990). Plasma samples and culture supernatants were defatted with an equivalent volume of FREON before chromatography. To protect the columns from excessive protein build up, plasma samples were diluted to between 10-50 % prior to being chromatographed. The samples were then acidified to pH 2.8 with glacial acetic acid and chromatographed on a Waters Protein Pak 125 column (Waters/Millipore, Lane Cove, NSW, Australia) in mobile phase (0.2M acetic acid, 0.05M triethylamine and 0.5% (v/v) Tween 20). Fractions were collected at 0.25 minute intervals (0.25 ml) and 50 µl aliquots of each fraction were assayed for the presence of IGF. Where multiple samples were to processed simultaneously, 4 broad pools of eluent were collected from the chromatography using a programmable Gilson fraction collector .

The measurement of IGF-I in fractions from the chromatography was determined using the radioimmunoassay described by Owens et al., (1990). Fractions from the Waters Protein-Pak column or standards (recombinant hIGF-I) were assayed by the addition of 30 µl Tris HCl pH 7.5 (0.4M), 200 µl RIA buffer (30mM NaH_2PO_4, 0.2 % (w/v) protamine sulphate, 10mM disodium EDTA, 0.2 % (w/v) NaN_3 and 0.05 % (v/v) Tween 20 at pH 7.5), ^{125}I-IGF-I (20,000 cpm)(50 µl) and 50 µl anti-IGF-I antibody (either a mouse monoclonal or rabbit polyclonal antibody) in RIA buffer (1/20,000 or 1/40,000 final dilution respectively). After 18 hours at 4 ^{o}C, 50 µl goat anti-rabbit or sheep anti-mouse serum (1/20) and 50 µl of either mouse or rabbit serum (1/250) were added to each sample for 1 hour at 4 ^{o}C. Ice-cold PEG 6000 (1ml, 6 % w/v in 150 mM NaCl) was then added and the tubes were centrifuged at 4000 rpm for 30 minutes at 4 ^{o}C. The supernatants were aspirated and the pellet counted in a gamma counter. The minimal detectable concentration fluctuated between individual assays (and is given in the results section for particular assays) due to variations in the standard curves for each assay on which the IGF-I determinations for unknowns were calculated. Also the limit of detection was much lower for assays employing the polyclonal antibody. The binding of tracer to the antibodies was between 20-30 % of total counts used for all assays carried out.

6.3.22 Radioimmunoassay for pGH

This assay was carried out on plasma collected from rats at weaning and employed a procedure similar to that previously described by Michalska (1988). The assay was carried out with a rabbit antiserum to pig pituitary GH (UCB-i571, Brussels, Belgium), porcine growth hormone as a standard and [^{125} I]-iodo-pGH as radioligand. Porcine GH was iodinated to specific activities between 80 and 100 Ci/g using chloramine T (Van Obberghen-Schilling et al., 1983) and purified as described by Francis et al., 1989). The routine pGH RIA was performed in 0.05 M sodium phosphate buffer containing 5g/l BSA at pH 7.4. Replicate polystyrene tubes containing approximately 30,000 cpm [^{125}I]-pGH, rabbit anti-pGH serum diluted 1/20 and triplicate 20 µl plasma samples were incubated at room temperature for 16-20 hours (the final reaction volume was 0.3 ml). After incubation, cellulose coated donkey anti-rabbit immunoglobulin (Sac-Cell, Unsworth Hall,Washington)(100 µl) was added, the tubes mixed and incubated for 30 minutes at room temperature. Subsequently, 0.5 ml of sodium phosphate buffer was added and the tubes centrifuged at 3000 x g for 10 minutes. The supernatants were then aspirated and the radioactivity in the pellet was counted in a gamma scintillation spectrometer.

In each assay a series of controls were included to ensure the assay was valid and to enable construction of a standard curve for determining the concentration of pGH in the plasma samples. Triplicate blank tubes were set up in which the anti-pGH antibody was omitted. Radioactivity in the pellets from these tubes represents non-specific binding of the tracer to the tube and was subtracted from all values. A second set of triplicate tubes included in each assay contained everything apart from pGH. These reference tubes determine the amount of tracer binding to the antibody in the absence of competing ligand. A final series of triplicate tubes contained dilutions of pGH (3.9-2000 pg/tube) and enabled a standard curve to be derived for each assay. Plasma pGH results given in this thesis are means of triplicate determinations on each sample. The limit of detection of the pGH assay varied from 7 to 17 ng/ml. Immunoreactive pGH in plasma from normal rats was lower than the sensitivity of the assay, indicating the low cross-reactivity of the antibody used with rGH and confirming the specificity of the assay for pGH.

6.3.23 Western ligand blotting

Proteins were separated by 12.5% (w/v) SDS-polyacrylamide electrophoresis under non-reducing conditions as described by Laemmli (1970). They were transferred to nitrocellulose at 300mA for 3 hours using a Hoeffer Transphor TE24 apparatus (Hoeffer Scientific, San Francisco, CA, USA.). Ligand blots were performed as outlined by Hossenlopp *et. al.* (1986). Briefly, nitrocellulose was washed with 0.15 M NaCl, 0.1 M Tris-HCl pH 7.4, 0.05 mg/ml sodium azide containing 3% (v/v) Nonidet P40 for 10 minutes followed by a 2 hour wash in the same buffer containing 1% (v/v) RIA grade BSA and a 10 minute wash in buffer containing 1% (v/v) Tween 20. The filters were incubated with 1 x 10^6 cpm ^{125}I-IGF-II overnight at 4 ^{o}C in buffer containing 1% (v/v) BSA and 1% (v/v) Tween 20. Following 2 x 45 minute washes in buffer to remove unbound tracer, filters were exposed to X-ray film (A8323, Konica, Tokyo, Japan) for 11-28 days at -80 ^{o}C with 2 intensifying screens.

6.3.24 Microinjection and generation of transgenic animals

All of this work was carried out by Mr. Du Zhang Tao (Dept. of Obstetrics and Gynaecology, University of Adelaide, S.A.). Immature female Hooded Wistar rats (4 weeks old) were injected with 15 IU pregnant mare serum gonadotropin (PMSG) at twelve noon on day -2 followed 48 hours later by 15 IU human chorionic gonadotropin (HCG). They were then mated with intact males of the same strain. On the following day, mated females were killed by cervical dislocation, 23-24 hours after the HCG injection. Fertilised eggs were recovered by opening ampullae of the oviducts in a petri dish containing 2 ml HEPES-HTF medium supplemented with BSA (5 mg/ml) and hyaluronidase (300 IU/ml). As soon as the eggs had been separated from cumulus cells, they were picked up and washed twice in modified rat embryo culture medium (Zhang et al., 1990). The eggs were then placed in microdrops of the culture medium overlayed with paraffin oil and incubated at 37 ^{o}C under 5 % CO_2, 5 % O_2, 90 % N_2 (v/v) for 1 or 2 hours before microinjection.

For microinjection, 20-30 eggs were placed into a drop of HEPES-HTF medium overlayed with paraffin oil in the injection chamber. An aliquot of the DNA solution was centrifuged for 5 minutes at 10,000 rpm before use to prevent the injection pipettes from clogging due to residual glass beads from the geneclean^{TM} procedure. DNA solution (2-3

pl) was injected into each male pronucleus. When the eggs were injected , they were transferred back to the microdrops of culture medium and cultured for 1 or 2 hours.

Synchronisation of pseudopregnant recipients was made by pairing 12-14 week old virgin female rats with vasectomised male rats of the same strain the day before microinjection. Matings were determined by checking for the presence of vaginal plugs the next day. When all eggs were injected in the afternoon, healthy injected eggs were selected for transfer and placed in microdrops of HEPES-HTFoverlayed with paraffin oil. Recipients were anaesthetised with tribromoethanol solution (2 %, 0.015 mg/g body weight) and embryo transfer carried out with procedures similar to those described by Walton et al., (1983). Twelve to twenty single cell embryos were transferred to each oviduct of the rats.

6.3.25 Miscellaneous items

i) Collection, Handling and storage of plasma.

Rats were anaesthetised with ether and blood samples collected from the tail vein into tubes containing 0.1 M EDTA. Samples were kept on ice for 2-3 hours after which time plasma was recovered by cenrifuging the samples at 2000 x g for 30 minutes at 4 ^{o}C. Plasma aliquots were stored at -20 ^{o}C until further use.

ii) Densitometric quantitation of bands on autoradiograms

Quantitation of bands on autoradiograms was performed on a Molecular Dynamics laser densitometer using the IMAGEQUANT package. Exposure times were adjusted so that the signals were within the linear range of the film used.

iii) Computer Programs

ANALYSEQ, a suite of DNA analysis programmes (Staden, 1982) was used to identify restriction enzyme sites, translate coding regions into amino acid sequence and produce sequence data of the complementary strand.

Sequence databases GenBank, EMBL, NBRF and VECBase were screened using the suite of programs from Genetics Computing Group of the University of Wisconsin (Devereux *et al.*, 1984) particularly FASTA.

PRIMER was used to design suitable PCR primers that did not hybridise to each other or to repetitive sequences within the mouse or rat genome.

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