EXPRESSION OF PORCINE GROWTH HORMONE IN BACTERIA AND TRANSGENIC ANIMALS

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by

Peter Darren Vize, B.Sc. (Hons.)

Department of Biochemistry
University of Adelaide
Adelaide, South Australia.

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SUMMARY

The aim of the work described in this thesis was to utilize gene manipulation techniques to improve animal growth. Two different approaches were undertaken. The first approach was to achieve the efficient expression of porcine growth hormone (PGH) in *E.coli*, so as to provide a large source of material for use in the animal industry. The second was to fuse the PGH gene to a heterologous promoter and introduce the fusion gene into the germ line of transgenic animals. Initially transgenic mice were used to determine the effectiveness of the fusion gene in promoting growth, followed by later experiments which introduced the gene into transgenic pigs to examine the possibility of improving the growth performance of farm animals. The results obtained during the course of this work are listed below:

A porcine pituitary cDNA library was constructed, and an oligonucleotide probe used to isolate a PGH cDNA clone. This clone was completely sequenced, and subsequently used to screen a porcine cosmid library. Screening the library resulted in the isolation of a PGH genomic gene, which was also completely sequenced.

The PGH cDNA clone was cloned into a bacterial expression vector and the resulting plasmid assayed for PGH production in *E.coli*. This construct was found to express high levels of PGH mRNA, but produced no PGH protein. Oligonucleotide-directed mutagenesis was used to construct a number of plasmids which differed from the original in either 5' non-coding or 5' coding sequences. The alteration of 5' non-coding sequences was found to result in the high level production of PGH protein.

The human metallothionein promoter (hMT-IIA) was joined to PGH gene sequences and introduced into both transgenic mice and transgenic pigs. Most of the transgenic mice containing the fusion gene were found to grow

at vastly increased rates, up to twice that of their non-transgenic littermates. These mice were shown to pass on the foreign gene and the large growth phenotype to a portion of their offspring. Also, at least one of the transgenic pigs grew at a significantly faster rate than control littermates, illustrating for the first time the feasibility of producing transgenic farm animals with enhanced growth characteristics.

Modifications to the human metallothionein promoter by oligonucleotide directed mutagenesis were performed in an attempt to reduce the high level expression observed from the un-induced promoter in both transgenic mice and transgenic pigs. A construct containing an altered promoter linked to the PGH gene was introduced into transgenic mice, whose growth was then studied on both normal and zinc containing diets. The analysis of a second altered promoter is in progress.

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, it contains no material which has been previously published except where due reference is made in the text.

PETER DARREN VIZE

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CHAPTER 1

INTRODUCTION:

THE REGULATION AND MANIPULATION OF GROWTH

1.1 Introduction

The regulation of growth in mammals is governed by the complex interaction between nutritional and hormonal status. Advances in understanding the biology of growth over the past 60 years have allowed its manipulation in a number of different ways. The first approach used was to isolate and inject protein fractions containing growth promoting hormones into animals (Evans and Long, 1921). This approach has been modified by procedures allowing the isolation of pure proteins and more recently via the expression and purification of eukaryotic hormones from recombinant microorganisms. The development and sophistication of molecular biological techniques has also recently allowed the manipulation of the genes encoding growth stimulating hormones and provided mechanisms of incorporating altered genes back into the germ line of animals (Jaenisch and Mintz, 1974; Gordon *et al.*, 1980), providing a second approach to the manipulation of growth. The work described in this thesis utilized both of these approaches with the eventual aim of improving farm animal growth. Towards this end, recombinant DNA techniques were used to isolate and manipulate the gene encoding porcine growth hormone, so as to allow the production of large amounts of this protein in the bacterium *Escherichia coli*, for use in the animal industry. The PGH gene was also fused to a heterologous promoter and introduced into the germ line of both transgenic mice and pigs, to produce animals which over-express growth hormone and grow at increased rates.

In this Chapter the mechanisms by which growth is hormonally regulated and the approaches which have been used to alter growth will be discussed.

1.2 Hormonal regulation of growth

Growth in vertebrates is governed by a large number of different

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hormones produced in many different tissues. The major hormones responsible for the stimulation of growth are the members of the growth hormone cascade (see Figure 1-1). The cascade begins in the hypothalamus, where neurotransmitters stimulate the release of growth hormone releasing factor (GHRF, or somatocrinin), a peptide of about 44 amino acids in length. GHRF is transported to the pituitary via a network of portal capillary vessels, which responds by synthesizing and secreting growth hormone (GH, or somatotropin), a peptide hormone of around 190 amino acids in length. GH is transported through the circulatory system to the liver, where it induces the production of insulin-like growth factor-I (IGF-I, or somatomedin C) which induces linear growth. A number of questions exist as to exactly what role GH and IGF-I each play in stimulting growth, and this will be discussed in more detail below.

Other hormones involved in the regulation of growth include insulin, somatostatin, thyroid hormone (3,5,3'-L-triiodothyronine), androgens, corticosteroids and estrogens (for review see Daughaday et al., 1975a). Some of these hormones affect growth by interacting directly with members of the growth hormone cascade with the end result of reducing growth. A particularly clear example of this is the peptide hormone somatostatin, the production of which by the hypothalamus directly inhibits the release of GH from the pituitary. Others, in particular insulin and thyroid hormone, are involved in the maintenance of normal growth levels (Daughaday et al., 1975a).

1.2.1 Growth hormone releasing factor

i) Actions

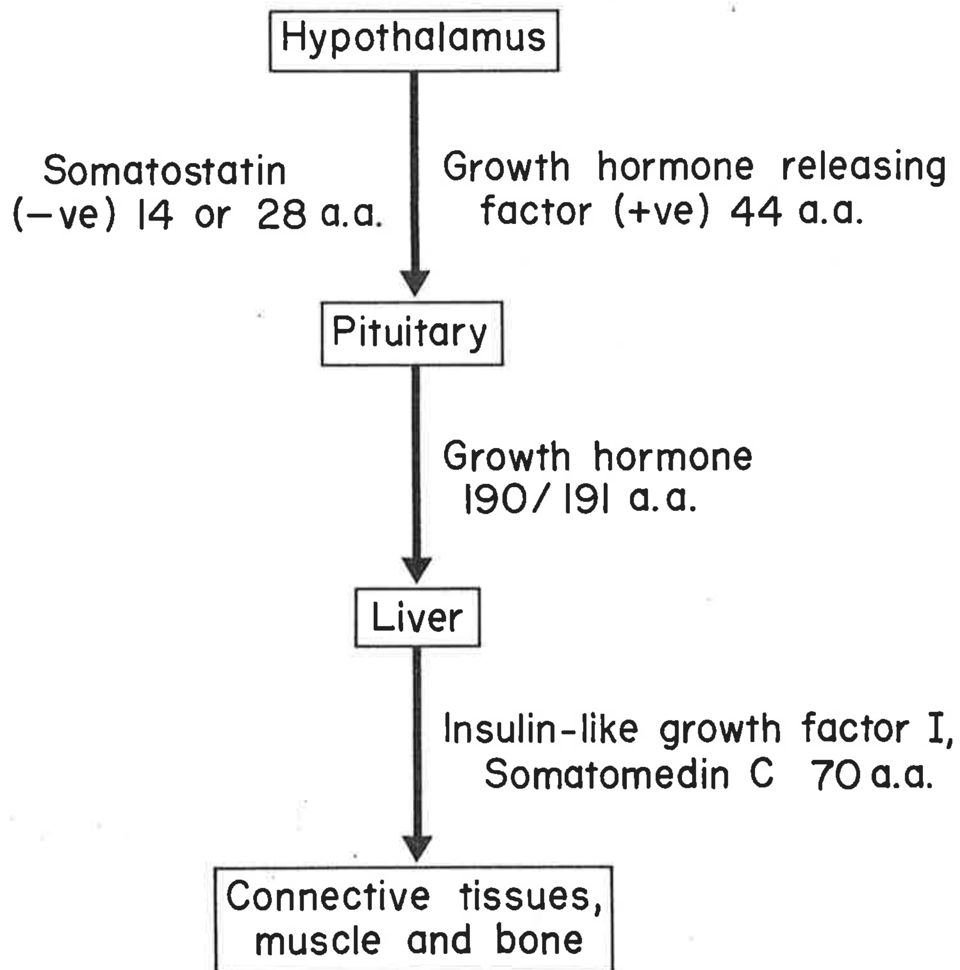
The hypothalamus produces a number of short peptide hormones whose purpose is to regulate the production and release of hormones from the pituitary. These include the tripeptide thyrotropin releasing factor (TRF)

FIGURE 1

The growth hormone cascade

The hormones involved in the growth hormone cascade are illustrated (adapted from Palmiter *et al.*, 1983).

overhead (1)



which regulates both thyrotropin and prolactin, the 10 amino acid peptide gonadotropin releasing factor (GRF), the 14 or 28 amino acid peptide somatostatin, the 41 amino acid peptide corticotropin releasing factor (CRF) and, GHRF. Each of these peptides, with the exception of somatostatin, contains an amidated carboxyl-terminus. Each of these releasing factors are quite specific in that they induce the pituitary to release only the appropriate hormone or hormones (for review see Ling *et al.*, 1985).

Growth hormone releasing factor is produced in response to neurotransmitters (reviewed in Frohman and Jansson, 1986). GHRF stimulates both the synthesis, at the transcriptional level (Barinaga *et al.*, 1983), and the release (Barinaga *et al.*, 1985) of GH from the pituitary at extremely low concentrations (10^{-13} M, Brazeau *et al.*, 1982a). This stimulation of GH release is specifically blocked by somatostatin in a noncompetitive fashion (Brazeau *et al.*, 1982a). IGF-I, the final hormone of the growth hormone cascade, both reduces the response of the pituitary to GHRF (Brazeau *et al.*, 1982b; Abe *et al.*, 1983) and stimulates the hypothalamus to produce somatostatin (Berelowitz *et al.*, 1981).

GHRF stimulates the synthesis of GH by interacting with specific receptors on the pituitary (Seifert *et al.*, 1985) which stimulates the production of cyclic-AMP (Bilezikjian and Vale, 1983). Raising the intracellular concentration of c-AMP by other means also induces the production of GH (Brazeau *et al.*, 1982c).

ii) Structure

GHRF is a peptide hormone of 43 or 44 amino acids in length produced by the hypothalamus. The hormone was first purified from pancreatic tumours which were isolated from human acromegalics. The protein was found to be 44 amino acids long and contain an amidated carboxyl-terminus (Guillemin *et al.*, 1982; Rivier *et al.*, 1982). Antibodies raised against the pancreatic

hormone were utilized to purify GHRF from the hypothalami of a number of animals by immuno-affinity chromatography (see below). Human hypothalmic GHRF was found to be identical to the pancreatic peptide, including the amidated carboxyl-terminus (Ling et al., 1984). GHRFs from a number of animals were found to be very similar in sequence, with the first 27 amino acids being identical in human (Guillemin et al., 1982), porcine (Bohlen et al., 1983), bovine (Esch et al., 1983), and caprine (Brazeau et al., 1984) hormones. The only studied GHRF which differed significantly in structure from the human hormone was that of the rat (Spiess et al., 1983), which was found to be 43 amino acids in length and contained a number of additional amino acid substitutions. This peptide was also found to possess a free carboxyl-terminus (Spiess et al., 1983).

The high conservation of sequence between the GHRFs of a number of organisms is reflected in the wide cross-species activity of this hormone. Human GHRF has been shown to actively stimulate the release of growth hormone in animals as diverse as chickens (Leung and Taylor, 1983) and goldfish (Peter et al., 1984).

iii) Gene organization

The first GHRF DNA sequences isolated were human cDNAs, detected in libraries constructed from GHRF producing pancreatic tumor RNA, utilizing synthetic oligonucleotide (Gubler et al., 1983) and antibody (Mayo et al., 1983) detection systems. A rat GHRF cDNA has also been isolated, by utilizing the human GHRF cDNA as the hybridization probe (Mayo et al., 1985a). Both human and rat cDNA clones were found to encode precursor hormones, which contained both amino- and carboxyl- terminal extensions in addition to a hydrophobic signal peptide. The amino-terminal extensions were approximately 7 amino acids long, and were separated from the mature peptide sequence by an arg-arg basic cleavage site. The human GHRF precursor has a

carboxyl-terminal extension of either 30 or 31 amino acids in length, depending on the presence or absence of a serine at residue 103. The rat GHRF precursor also has a carboxyl-terminal extension of 30 amino acids. The variation in the length of the human carboxyl-terminal extension arises from the alternative splicing of the GHRF mRNA (discussed below). In the human, but not the rat GHRF precursor the junction between the carboxyl-terminal extension and the mature peptide occurs at the amidation signal, leu-gly (Bradbury et al., 1982), consistent with amino acid analysis which indicates that rat GHRF is not amidated (Spiess et al., 1983).

Southern analysis of human DNA using a GHRF cDNA probe indicates that only a single copy of the GHRF gene exists per haploid chromosome complement in humans (Mayo et al., 1983).

The cDNA clones have both been used to isolate their corresponding genomic genes (Mayo et al., 1985a; Mayo et al., 1985b). The human and rat genes were both found to cover approximately 10 kb of genomic DNA, and each contained four introns. The first three of these introns are of similar size and are found in identical positions in the two genes. The fourth intron, which splits the region encoding the carboxyl-terminal extension, is found in a different position. Interestingly, the point at which the fourth intron is present coincides with the point at which the amino acid sequences of the rat and human carboxyl-terminal extensions lose homology (Mayo et al., 1985b) indicating that some alteration of the splicing pattern is responsible for the sequence divergence. Examination of the nucleotide sequence of the human gene indicates that the alternative splicing of this fourth intron is responsible for the presence of two different GHRF precursors (Mayo et al., 1983). The different forms arise from the alternative usage of two consensus splice sites at the 5' end of exon 5 (Mayo et al., 1985b).

1.2.2 Growth hormone

Growth hormone is a member of a family of related proteins which includes prolactin, and in humans, placental lactogen (HPL, or somatomammotropin, see Wallis, 1981 for review).

i) Actions

The connection between the pituitary and the regulation of growth was first made from the observation that acromegalic patients contained enlarged pituitaries (Marie, 1889). A more direct illustration of the growth promoting effects of pituitary hormones was made by Evans and Long (1921) who produced giant rats by the intraperitoneal injection of anterior pituitary extracts. The destruction of the pituitary, and thus of GH production, by hypophysectomy causes a reduction in growth rate which can be alleviated by the injection of growth hormone (Smith, 1927). The injection of GH into humans suffering from pituitary dwarfism has been shown to stimulate linear growth (Raben, 1958). In recent years bacterially synthesized GH (human and bovine) has been purified and shown to be equally as efficient in inducing growth as hormone isolated from tissue (Olson *et al.*, 1981; Hintz *et al.*, 1982; Hart *et al*, 1984; Kaplan *et al.*, 1986).

Growth in response to the administration of GH is the result of a wide range of metabolic effects, including increasing amino acid uptake and protein synthesis, both insulin-like and insulin-antagonistic effects on carbohydrate metabolism, and stimulating the hydrolysis of lipids (see Franchimont and Burger, 1975 for review). The early observation that serum from normal rats, or from hypophysectomized rats previously injected with GH, could stimulate the uptake of ^{35}S into incubated cartilage while serum from hypophysectomized animals supplemented with GH *in vitro* could not, indicated that there was some additional factor present in serum, produced in response to GH which mediated cartilage growth (Salmon and Daughaday,

1957). This theory has since been shown to be correct, and while GH may have some intrinsic growth promoting properties of its own (Isaksson *et al.*, 1982; Madsen *et al.*, 1983; Mittra, 1984; Isaksson *et al.*, 1985), its growth promoting properties are thought to mostly be due to the stimulation of IGF-I production (see Froesch *et al.*, 1985 for review).

The correlation between circulating IGF-I and GH levels was demonstrated by the measurement of IGF-I levels in patients with either abnormally high or low GH levels. IGF-I levels were found to be low in pituitary dwarfs and in hypophysectomized animals, and very high in patients suffering from acromegaly (Zapf *et al.*, 1980). The depressed levels observed in hypophysectomized animals can be elevated by treatment with GH (Kaufmann *et al.*, 1978). Recently the level of circulating GH has been shown to regulate the level of IGF-I mRNA present in the liver (Roberts *et al.*, 1986).

ii) Structure

Growth hormone is a polypeptide hormone of approximately 190 amino acids in length which contains two disulphide bridges (Li *et al.*, 1969; Niall, 1971). The hormone is synthesized in the pituitary as a pre-hormone containing a hydrophobic signal peptide which is removed during secretion from the cell (Sussman *et al.*, 1976; Seeburg *et al.*, 1977). Growth hormone has been purified and sequenced from a number of organisms, including human, bovine, ovine, porcine and equine species (see Dayhoff, 1972 for review). In addition the amino acid sequences of the GH of a number of other animals has been deduced from the analysis of cDNA clones (see below).

A number of GH proteins have been detected which differ from the major (190 amino acid) 22,000 molecular weight single chain form. These include aggregates, inter-chain disulphide dimers and proteolytically modified forms (for review see Lewis, 1984). The most common, and perhaps the most interesting variant is the 20,000 molecular weight form (20 K) of human GH

(Lewis *et al.*, 1978), which lacks amino acid residues 32 to 46 of the normal protein (Lewis *et al.*, 1980). Analysis of the human GH gene sequence has indicated that the 20 K form arises from the alternative splicing of the GH mRNA which utilizes a cryptic splice site within exon 3 of the gene (Wallis, 1980; DeNoto *et al.*, 1981). The 20 K form makes up approximately 10% of the GH present in the human pituitary (Lewis *et al.*, 1978) and has been shown to be fully active in promoting growth, but appears to have a lowered affinity for GH receptors (Sigel *et al.*, 1981). The deletion of 15 amino acids from the normal GH molecule alters some aspects of its activity, with the variant hormone lacking some of the insulin-antagonistic (diabetogenic) effects on carbohydrate metabolism associated with the full hormone (Frigeri *et al.*, 1979).

iii) Gene Organization

The cDNA encoding the rat GH gene was one of the first characterized eukaryotic genes (Seeburg *et al.*, 1977). Since then cDNA clones encoding part or all of human (Roskam and Rougeon, 1979; Martial *et al.*, 1979), bovine (Miller *et al.*, 1980), porcine (Seeburg *et al.*, 1983), chicken (Souza *et al.*, 1984), murine (Linzer and Talamantes, 1985), and salmon (Sekine *et al.*, 1985) growth hormones have been isolated from pituitary cDNA libraries. In each case the clones encoded precursors of around 216 amino acids in length, incorporating a signal peptide of 22-26 amino acids and a mature GH protein of 188-191 amino acids.

The cDNA probes have been used to isolate the genomic genes encoding rat (Page *et al.*, 1981; Barta *et al.*, 1981), human (DeNoto *et al.*, 1981) and bovine (Woychik *et al.*, 1982) GH. Each of these genes contain four introns, which are present in identical positions and are of similar lengths. The single exception to the high conservation of intron length observed in these genes is the second intron of the rat gene, which is 500

bp longer than the eqivalent introns in human and bovine GH genes. The increase in length is due to the insertion of a number of copies of a short repeated sequence which possesses RNA polymerase III promoter activity and is present in many copies in the rat genome (Gutierrez-Hartmann *et al.*, 1984).

Southern analysis of both rat (Page *et al.*, 1981) and bovine (Woychik *et al.*, 1982) DNA has indicated that both these animals contain a single copy of the GH gene per haploid genome. Nucleotide sequence data and cosmid mapping has revealed that there are five GH related genes in the human genome (Seeburg, 1982; Barsh *et al.*, 1983). Three of these genes encode placental lactogen (HPL) proteins while two encode GH proteins. Only one of the two GH genes codes for a protein identical in sequence to the previously studied human protein (Niall, 1971) and cDNA (Roskam and Rougeon, 1979; Martial *et al.*, 1979) sequences. The second gene encodes a GH-like protein known as HGH-V (variant), which contains fifteen amino acid residues which differ from the HGH-N (normal) sequence (Seeburg, 1982). There is no evidence for the HGH-V gene being expressed *in vivo*, although it does contain a promoter which is active when cloned next to the SV40 enhancer and transfected into tissue culture cells (Pavlakis *et al.*, 1981).

iv) *GH gene regulation*

The regulation of GH gene transcription has been studied for many years using transformed pituitary cell lines. The most widely studied GH gene is that of the rat, which is therefore given emphasis here.

The rat GH gene is regulated in a tissue-specific manner, with transcription being limited to a subset of cells within the pituitary known as somatotrophes (Daughaday, 1981). Expression from the GH gene is rapidly swithed off in pituitary/fibroblast fusion cells (Strobl *et al.*, 1984) and usually is expressed upon transfection only in pituitary derived cell lines

(Crew and Spindler, 1986; Nelson *et al.*, 1986). In fact, stable transformants are often only obtained when pituitary cell lines are used. The human GH gene differs from the rat GH gene in this respect as it is expressed when transfected into non-pituitary cell lines (Robins *et al.*, 1982; Robins and Seeburg, 1982). The expression of the rat GH gene is controlled by a tissue-specific enhancer, present between bases -235 and -181, which is capable of conferring pituitary-specific expression on heterologous genes in an orientation independent fashion (Nelson *et al.*, 1986).

In addition to GHRF (1.2.1), GH gene transcription is also stimulated by a number of other factors including thyroid hormone (Spindler *et al.*, 1982) and the synthetic glucocorticoid, dexamethasone (Yu *et al.*, 1977; Spindler *et al.*, 1982). The magnitude of the transcriptional response to these hormones is in the order of three fold for thyroid hormone and five fold for dexamethasone plus thyroid hormone (Diamond and Goodman, 1985). The induced levels of GH mRNA accumulate to much higher levels than expected from this increase in transcription, indicating that dexamethasone and thyroid hormone may also influence GH mRNA stability in addition to GH gene expression (Diamond and Goodman, 1985). The promoter sequences responsible for regulation by thyroid hormone have been localized to between -202 and -183 of the rat gene (Crew and Spindler, 1986; Larsen *et al.*, 1986), in the same region as the tissue-specific GH enhancer (-235 to -181, Nelson *et al.*, 1986). The induction by thyroid hormone is accompanied by alterations in the chromatin structure within the promoter, around the -171 area (Nyborg and Spindler, 1986). The sites responsible for binding the glucocorticoid receptor have been localized in the human GH gene. There appears to be two such sites, one within the first intron of the gene (Moore *et al.*, 1985) and one within the first 290 bp of the promoter (Eliard *et al.*, 1985). The site within the first intron has been shown to be capable of

conferring glucocorticoid responsiveness to a heterologous promoter (Slater *et al.*, 1985).

1.2.3 Insulin-like growth factor-I

i) Actions

As disscussed above (1.2.2.i), IGF-I is synthesized, predominantly in the liver (Daughaday *et al.*, 1975b; Spencer, 1979) in response to the presence of circulating GH (1.2.2). The production of IGF-I is developmentally regulated, with IGF-I being present in low levels in the early neonate and rising to adult levels in the rat at around four weeks of age (Sara *et al.*, 1980; Daughaday *et al.*, 1982; Adams *et al.*, 1983). The structurally related peptide, IGF-II, is regulated in the opposite fashion, with levels being highest in the foetus, and dropping rapidly shortly after birth (Moses *et al.*, 1980).

Insulin-like growth factor-I has a wide range of metabolic actions. These include the stimulation of DNA synthesis and cell division in cultured cells (including chick embryo fibroblasts (Rechler *et al.*, 1978), chondrocytes (Froesch *et al.*, 1976) and bone cells (Schmid *et al.*, 1983a)), the stimulation of protein synthesis (Hall, 1972; Froesch *et al.*, 1976) and enhancing the uptake and metabolism of glucose (Ewton and Florini, 1980; Zapf *et al.*, 1981a). Some of the effects on metabolism which are mediated by IGF-I, such as increasing the uptake of glucose, may be due to the cross-reaction of IGF-I with the insulin receptor (Zapf *et al.*, 1981b).

The direct effect of IGF-1 on growth has been demonstrated in hypophysectomized rats, where the administration of IGF-I has been shown to stimulate growth in a dose dependent manner (Schoenle *et al.*, 1982). More recently the availability of large amounts of recombinant IGF-I has enabled the demonstration of the growth promoting effects of this hormone in normal rats (Hizuka *et al.*, 1986) and in insulin deficient rats (Scheiwiller *et*

_al._, 1986).

Insulin-like growth factors present in serum are found bound to high molecular weight carrier proteins. The half life of unbound IGF-I in serum is approximately 20 minutes, while IGF-I bound to the 180 K carrier protein, the major active form of carrier (Moses _et al._, 1979), has a half life of two to four hours (Zapf _et al._, 1985). The synthesis of the 180 K carrier protein is directly regulated by the level of circulating GH (Moses _et al._, 1976; Kaufmann _et al._, 1978; Schoenle _et al._, 1985).

The difficulty in demonstrating the direct growth promoting effects of IGF-I are therefore not surprising considering the short half life of unbound IGF. As discussed above the direct effects of IGF-I on normal as opposed to hypophysectomized animals (where the doses required are much smaller) could not be demonstrated until recombinant DNA techniques enabled the production of large amounts of IGF-I from _E.coli_ (Hizuka _et al._, 1986).

ii) Structure

The protein sequence of IGF-I was first determined from IGF-I isolated from human serum. The hormone was found to be 70 amino acids long, and contains three disulphide bonds which are at positions analogous to those of insulin (Rinderknecht and Humbel, 1978). The amino acid sequence of IGF-I is very similar to that of pro-insulin, with the exception of the C chain which is removed during insulin secretion but which is present in the mature IGF-I molecule. Twenty five of the fifty one residues of the human insulin molecule are identical to those of IGF-I (Rinderknecht and Humbel, 1978). In addition to the C peptide, mature IGF-I also contains an additional eight amino acids on the carboxyl-terminus, which are not present in the insulin molecule.

In addition to insulin, IGF-I is also structurally related to a number of other hormones including IGF-II, which is thought to be involved in the

regulation of foetal growth (Adams _et al._, 1983), relaxin and nerve growth factor (see Bradshaw, 1980, and Blundell and Humbel, 1980 for reviews).

iii) Gene organization

The first IGF-I clone isolated was a cDNA sequence detected in a human foetal liver cDNA library using a synthetic oligonucleotide hybridization probe. Analysis of this clone indicated that like many other small peptide hormones, IGF-I is synthesized as a precursor molecule which contains a carboxyl-terminal extension of 35 amino acids in addition to a 22 or 25 amino acid amino-terminal signal peptide (Jansen _et al._, 1983). A recent report which described the isolation of a number of independent IGF-I cDNA clones from a human liver library has indicated that two different IGF-I precursors exist _in vivo_ (Rotwein, 1986). This paper describes the isolation of seven clones, two of which were identical to the cDNA described by Jansen _et al_. (1983) and are referred to as IGF-IA, and another five clones which encode IGF-I precursors (IGF-IB) which differ in the sequence of the carboxyl-terminal extension. The IGF-IB encoded proteins are identical to IGF-IA from their amino-terminus down to the lysine residue which is 16 amino acids past the carboxyl-terminus of the mature IGF-I peptide. Following this residue there is an additional 19 amino acids in IGF-IA and 61 amino acids in IGF-IB, which share no amino acid homology. Southern analysis of the human genome indicates that the IGF-I gene exists as a single copy per haploid genome (Brissenden _et al._, 1984), and, as the point of sequence divergence coincides with the position of an intron in the IGF-I gene (Rotwein _et al._, 1986; see below), the two different forms appear to arise from the alternative splicing of the IGF-I transcript (Rotwein, 1986).

Both cDNA and oligonucleotide probes have been used to isolate the IGF-I genomic gene from human gene libraries (Ullrich _et al._, 1984; Bell _et_

al., 1985; Rotwein et al., 1986). The gene was found to be over 45 kb in length and contains four introns which range in size from 1.4 to greater than 21 kb. The last two exons encode the different IGF-IB and IGF-IA carboxyl-terminal extensions respectively, and each of these exons are followed by polyadenylation signals (Rotwein et al., 1986). The positions of the introns are analogous to those found in IGF-II genes (Dull et al., 1984), and to those within the genes encoding the structurally related proteins insulin and relaxin, with the exception of intron 3, which is not present in the latter two genes (Bell et al., 1980; Hudson et al., 1983).

1.3 Altering growth by hormone therapy

All three of the hormones involved in the growth hormone cascade (Figure 1-1) have been successfully used to induce growth directly, by the injection or infusion of purified hormone. This section describes the current state of knowledge of the effects of each hormone and its usefullness in growth therapy.

1.3.1 Growth hormone releasing factor

Due to its small size (43/44 amino acids, 1.2.1.ii) much attention has been focussed on GHRF as a possible substitute for GH for the treatment of growth disorders. The small size of the molecule makes it possible to obtain large amounts by chemical synthesis. Also, GHRF is active at very low concentrations *in vivo* (Brazeau et al., 1982a), which may make it more efficient in clinical applications than GH. In addition, GHRF has been shown to be active when administered by intranasal inhalation (Evans et al., 1983).

The small size of the GHRF molecule has allowed the activity of a number of human GHRF analogues to be studied by chemically synthesizing a range of derivatives. Deletion of carboxyl-terminal residues has shown that

only residues 1-29 are required for almost full biological potency in vivo, as long as the molecule contains an amidated carboxyl-terminus (Rivier et al., 1982; Wehrenberg and Ling, 1983). In contrast to the deletion of carboxyl-terminal sequences, the deletion of even a single amino-terminal residue was found to drastically reduce biological potency (Guillemin et al., 1982). There have been no reports of the expression of GHRF in bacterial systems.

Although human GHRF, and its synthetic analogues and derivatives have been shown to be capable of inducing the release of GH both in vivo and in vitro (reviewed in Frohman and Jansson, 1986), its ability to induce growth is much less simple to demonstrate. The administration of human GHRF by continuous infusion initially induces a rapid increase in serum GH levels, which gradually decline to a much lower concentration over the following 24 hours (Wehrenberg et al., 1984; Losa et al., 1984). Following this reduction in serum GH level, the response to further doses of GHRF is greatly reduced, which is mediated at least in part by the down-regulation of GHRF receptors (Bilezikjian et al., 1986).

Experiments which directly compared the ability of continuously infused versus intermittent pulsatile administration have indicated that only the latter form of administration results in increased growth in either normal or GHRF deficient rats (Clark and Robinson, 1985). This is probably a direct reflection on the increased effectiveness of GH in inducing growth when released in a pulsatile, rather than a continuous fashion (reviewed in Jansson et al., 1985), and also on the increased GH content of pituitaries in response to pulsatile rather than constant exposure to GHRF (Clark and Robinson, 1985).

In summary, GHRF appears to be a potential alternative to GH for use as a growth promoting hormone. It is active at very low concentrations and

is easily produced by chemical synthesis.

1.3.2 Growth Hormone

The first illustration of the growth potentiating effects of GH were performed over sixty years ago (1.2.2.i) and have been applied clinically for many years. The first report of the successful treatment of human GH deficiency by the injection of human GH was made by Rabin in 1958. The use of this therapy for almost 30 years has provided a wealth of knowledge on the details of the response to human GH (reviewed by Frasier, 1983). The administration of human GH to patients suffering from pituitary dwarfism has been shown to increase both height and weight and also to alter body composition.

The application of GH as an anabolic agent in livestock production has been pursued for many years. The administration of bovine GH to young pigs was initially shown to increase the efficiency of food utilization and to produce leaner meat, but produced no effects on weight gain (Truman and Andrews, 1955). More recent studies which measured the effect of porcine GH on growing pigs indicated that injection of 0.13 mg/kg/day (Machlin, 1972) or 0.022 mg/kg/day (Chung et al., 1985) of PGH improved daily weight gain by 14 % and 10 % respectively, and also improved feed conversion indicating the potential of this approach in improving animal performance. In addition to improving growth rates, bovine GH has been shown to increase milk production in cattle by up to 41 % (Bauman et al., 1985).

Although the experiments of Machlin (1972) illustrated the potential for enhancing animal growth with GH, they also demonstrated that the injection of high levels of GH are toxic in the pig. The main symptoms of GH toxicity were liver and kidney degeneration and arthritis. The level of PGH found to cause these harmful effects was 0.22 mg/kg/day or greater. As only one tenth of this amount is required to enhance growth (Chung et al.,

1985), this is not a significant problem.

The production of recombinant GH in *E.coli* has provided an almost unlimited source of GH for use both in human clinical situations and for use in the animal industry. Human GH was one of the first non-fused eukaryotic proteins expressed in *E.coli* (Goeddel *et al.*, 1979) and the purified product has been examined in a number of extensive trials. The bacterial protein contains an additional amino-terminal methionine residue in place of the signal sequence normally present in the HGH precursor. The recombinant methionyl-human GH (m-HGH) has been shown to be biologically active, and to have an equivalent specific activity to pituitary HGH (Olson *et al.*, 1981). Clinical trials carried out over the past four years have indicated that m-HGH is identical in its actions to pituitary HGH, in terms of increasing growth rates and raising serum IGF-I concentrations (reviewed by Kaplan *et al.*, 1986). The m-HGH was found to induce the formation of anti-HGH antibodies at a higher rate than pituitary HGH (probably due to *E.coli* contaminants acting as an adjuvant) but this is not a serious problem as the production of antibodies does not interfere with the enhancement of growth (Kaplan *et al.*, 1986).

Recombinant forms of methionyl-bovine GH (m-BGH) have also been produced and although the hormone was found to have slightly different properties to pituitary BGH in *in vitro* assays, it had identical activity *in vivo* (Hart *et al.*, 1984). The m-BGH has been shown to be more efficient than pituitary derived BGH at increasing milk production, and equipotent in improving weight gain in dairy cows (Bauman *et al.*, 1985). The injection of recombinant forms of methionyl-chicken GH (m-CGH) has been shown to significantly increase growth rates in hypophysectomized rats (Souza *et al.*, 1984). Both recombinant salmon GH (Sekine *et al.*, 1985), m-CGH and m-BGH (Gill *et al.*, 1985) have been shown to stimulate the growth of salmon.

Although the production of bacterially synthesized porcine GH has been reported (Seeburg *et al.*, 1983) there have been no reports on its effectiveness in inducing growth.

In summary, the injection of GH, either pituitary or *E.coli* derived, has been shown to stimulate growth in a number of animals in a dose dependent manner. An exception to this dose dependent response was PGH, which although it induced growth at low concentrations, was found to be toxic in high doses.

1.3.3 Insulin-like growth factor-I

The use of IGF-I as an anabolic agent has not been evaluated to anywhere near the extent of GHRF or GH. This is due to the short half life of circulating IGF-I not bound to carrier proteins (1.2.3.i) and the difficulty of obtaining large enough amounts of pure material for analysis. GHRF can be chemically synthesized (Guillemin *et al.*, 1982) and GH can be isolated in large amounts from pituitary tissue. Advances in peptide synthesis technology, and bacterial expression systems have made large amounts of IGF-I available only in recent years.

Chemically synthesized IGF-I has been shown to be identical to IGF-I purified from serum in its ability to stimulate DNA synthesis in cultured cells and in radioreceptor- and radioimmuno-assays (Van Wyk *et al.*, 1984). IGF-I and its analogues have also been produced in a number of different bacterial expression systems, including *E.coli* and *Staphylococcus aureus* (Peters *et al.*, 1985; Buell *et al.*, 1985; Nilsson *et al.*, 1985). An analogue of IGF-I which has had the methionine residue at position 59 altered to a threonine, which allows the removal of the amino-terminal methionine by cyanogen bromide cleavage, has also been produced and shown to retain full biological activity by the same criterion used to test the chemically synthesized IGF-I (Peters *et al.*, 1985).

The infusion of IGF-I purified from serum has been shown to increase the growth of hypophysectomized rats at levels of approximately 0.43 mg/kg/day (Schoenle et al., 1982; 1985). This dose increased the concentration of circulating IGF-I from 1.9 ug/ul to 168 ug/ul (Schoenle et al., 1982), in fairly close agreement with the IGF-I level of 155 ug/ul found in normal rats (Scheiwiller et al., 1986). However as human IGF-I has only one third of the activity of the endogenous rat IGF-I (Schmid et al., 1983b), this figure indicates that in real terms the injected rats only had one third of the normal serum concentration of IGF-I. Higher doses (1.0 mg/kg/day) were found to result in a more normal serum IGF-I level and to promote growth more effectively (Schoenle et al., 1982). In insulin deficient rats the infusion of approximately 2.4 mg/kg/day of recombinant IGF-I was found to raise the serum IGF-I levels to near normal, allowing for cross reactivity, and to stimulate growth as efficiently as insulin (Scheiwiller et al., 1986). Recombinant IGF-I has also been shown to increase the growth rates and serum IGF-I levels of normal growing rats at a dose of approximately 2.0 mg/kg/day (Hizuka et al., 1986).

1.4 Transgenic Animals

1.4.1 The generation of transgenic mice

Transgenic mice were first generated by Jaenisch and Mintz (1974) by microinjecting SV40 DNA into mouse blastocysts which were then implanted into foster mothers. A proportion of the mice born (40 %) were found to contain SV40 DNA in a number of tissues. The first demonstration of the direct transfer of cloned DNA into mice was performed by Gordon et al. (1980). These authors microinjected a plasmid which contained cloned herpes simplex and SV40 viral sequences directly into the pro-nuclei of fertilized mouse eggs which were then transferred to pseudo-pregnant foster mothers.

Two of the seventy-eight animals which developed from such eggs were found to contain rearranged plasmid sequences, neither of which expressed detectable levels of thymidine kinase (tk) from the foreign DNA.

The parameters affecting the efficiency of the production of transgenic mice have been accurately defined since the first demonstration of the validity of the approach. Conditions which result in approximately 25 % of the mice born from injected eggs being transgenic have been described by Brinster _et al_. (1985).

Since the first demonstration of the transfer of cloned DNA into adult mice it has been demonstrated that transgenic mice usually contain the DNA integrated into the chromosomal DNA as tandem arrays, which can be transmitted to progeny in a Mendelian fashion (Constantini and Lacy, 1981; Palmiter _et al_., 1982a). Two clear exceptions to this normal pattern of integration have been reported. The introduction of either duplicated bovine papillomavirus (BPV) DNA (Lacey _et al_., 1986) or plasmids containing the polyoma virus large T antigen and viral origin of replication (Rassoulzadegan _et al_., 1986) have resulted in the production of circular extrachromosomal elements in transgenic mice. The BPV mice were found to contain integrated copies of the introduced plasmid in most tissues, and extrachromosomal copies in skin tumours. The mice containing the polyoma virus sequences contained only extrachromosomal copies of the plasmid, which were rearranged and contained fragments of mouse DNA, and which were inherited by between 80 and 100 % of progeny (Rassoulzadegan _et al_., 1986).

Experiments by Brinster _et al_. (1981) illustrated the validity of using hybrid genes to obtain expression of the introduced sequences. The expression of the tk gene in transgenic mice was achieved by fusing the coding portion of the viral tk gene to the mouse metallothionein-I (MT-I, Durnam _et al_., 1980) promoter. Seventy percent of the transgenic mice

obtained were found to contain detectable levels of tk activity. Mice which contained and expressed the fusion gene were found to pass on both the foreign DNA sequence and (usually) the ability to express it to one half of their offspring (Palmiter et al., 1982a). This approach has been extended in recent years to the study of the regulation of a diverse range of eukaryotic promoters, a technique of unprecedented power. An extremely elegant example of such experiments is provided by the work of Overbeek et al. (1985) who introduced a fusion gene consisting of the murine alpha A-crystallin promoter fused to the coding region of the bacterial chloramphenicol acetyltransferase (CAT) gene into transgenic mice. The expression of the bacterial protein was found to be identical to the pattern of expression of the endogenous gene, with expression occurring only in the eye tissues. In addition, the fusion gene was found to be appropriately developmentally regulated, with expression beginning at day 12.5 of development.

The study of the tissue-specific expression of introduced genes in transgenic mice has revealed that fusion genes are sometimes expressed in novel developmental patterns. Examples of such novel specificities include: 1) the high expression in the brain, and (relatively) low expression in the liver of mice carrying copies of a mouse MT-I promoter/ human hypoxanthine phosphoribosyltransferase (HPRT) gene (Stout et al., 1985); 2) the expression of MT-I/ rat GH and MT-I/ human GH genes in a range of neuronal cells, including the hypothalamus (Swanson et al., 1985), and; 3) the high expression of both MT-I/ human GH and MT-I/ rat somatostatin fusion genes in the gonadotroph cells of the anterior pituitary (Low et al., 1986). In each of these cases the expression of the foreign gene occurred at a rate many times greater than that of the endogenous MT RNA, which was under the

control of the same promoter. The high expression of these genes in inappropriate tissues must therefore be either a consequence of intragenic control sequences, or, the fusion of different gene sequences has produced novel patterns of expression. Each of these fusion genes contained the 3' untranslated region of the human GH gene to provide the sequences involved in polyadenylation, except for the MT-I/ rat GH construct which contained the rat GH 3' sequences. The possibility therefore exists that the 3' region of the GH gene is involved in the altered regulation of the fusion genes.

1.4.2 Improving growth in transgenic mice

The application of the transgenic approach to improving animal growth was first demonstrated by Palmiter et al. (1982b). These authors introduced a sequence consisting of the mouse metallothionein-I (MT-I) promoter fused to the rat growth hormone structural gene into transgenic mice, and found that animals which expressed the foreign gene grew at up to 1.87 times the size of their control littermates. A similar series of experiments which utilized a fusion gene containing the MT-I promoter joined to the human GH gene also resulted in vastly increased growth rates in the majority of the transgenic animals (Palmiter et al., 1983). In both of these series of experiments, transcription of the fusion gene occurred predominantly in the liver and resulted in very high concentrations of circulating GH. In the later study (Palmiter et al., 1983) this high level of GH was shown to be associated with increased serum IGF-I levels.

Recently transgenic mice carrying a fusion gene consisting of the promoter from the murine class I major histocompatibility (MHC) complex H-2K gene and the coding sequences of the human GH gene have been produced (Morello et al., 1986). These mice were also larger than normal, growing up to 2.37 times the size of control animals, with transcription from the

fusion gene occuring in all tissues tested. Transgenic mice carrying integrated copies of MT-I/bovine GH gene and human MT promoter (hMT-IIA)/human GH genes have also been produced (Hammer *et al.*, 1985a). Expression of both these hybrid genes resulted in increased growth. The transcriptional activity of the hMT-IIA/HGH construct was shown to be regulated by both heavy metals and glucocorticoids, in contrast to transgenic mice carrying MT-I fusion genes which have only been shown to be regulated by heavy metals (Palmiter *et al.*, 1982a, 1983).

The initial studies on the expression of MT-I/ GH fusion genes in transgenic mice indicated that high circulating levels of GH cause decreased fertility in females (Hammer *et al.*, 1984). However the results of Morello *et al.* (1986) indicate that their female mice were capable of producing offspring if *in vitro* fertilization techniques were used. It was concluded from this that the inability of giant transgenic females to produce offspring was possibly due to a behavioural, rather than physiological abnormality.

Studies on dwarf "little" (*lit/lit*) mice, a strain of mice possessing a hereditary deficiency in GH production which is caused by some form of receptor-associated resistance to GHRF (Jannson *et al.*, 1986), have shown that the introduction of MT-I/rat GH genes removes the requirement for control by GHRF and results in increased growth (Hammer *et al.*, 1984). The introduction of the fusion gene enabled transgenic *lit/lit* mice to grow to over twice normal mutant size. This enhanced growth response caused by the fusion gene also resulted in the restoration of male fertility, which is severely impaired in male *lit/lit* mice (Hammer *et al.*, 1984). The introduction of either rat or human GH genes under the control of their own promoters did not result in the enhanced growth of transgenic *lit/lit* mice (Hammer *et al.*, 1984).

The generation of transgenic mice carrying copies of a MT-I/ human GHRF fusion gene has also been reported (Hammer *et al.*, 1985b). The GHRF gene used in this construct was a mini-gene which contained only intron 2 and the GHRF precursor coding region (1.2.1.iii), this was done to reduce the size of the 10 kb genomic GHRF gene. Transgenic mice which expressed the fusion gene contained detectable human GHRF in their serum and grew at increased rates, up to 1.5 times those of control littermates. These mice passed on both the foreign gene sequence and the enhanced growth phenotype to one half of their offspring. It is interesting to note that transgenic females possessing the enhanced growth phenotype were fertile, in contrast to the experiments which expressed GH in transgenic mice (Hammer *et al.*, 1984).

1.4.3 Application of transgenic techniques to other animals

Although the potential of transgenesis to improve livestock production has been obvious since it was demonstrated that mice could be made to grow at twice the normal rate (1.4.2), the application of these techniques has been difficult. This is largely due to the difficulties encountered when attempting to collect, microinject and implant fertilized eggs from large farm animals. The major difficulty faced in applying the direct micro-injection technique to farm animals is the opacity of fertilized eggs, which interferes with the visualization of the pronuclei, which is essential for obtaining transgenic animals at a reasonable efficiency (Hammer *et al.*, 1985c; Brinster *et al.*, 1985). This problem has been overcome by the use of either interference contrast microscopy (Hammer *et al.*, 1985c) or by stratifying the cytoplasm of eggs by centrifugation (Wall *et al.*, 1985).

Using this approach Hammer *et al.* (1985c) have produced transgenic rabbits, sheep and pigs utilizing a MT-I/ human GH gene construct. The proportion of animals born which were transgenic, approximately 10 % of

rabbits and pigs and 1 % of sheep, was much lower than obtained with mice (25 to 30%). Southern analysis of transgenic rabbit and pig DNA indicated that these animals contained complete copies of the foreign gene which were integrated in either head to tail or head to head fashion. In a number of rabbits and pigs both human GH mRNA and protein were detected. The levels of circulating HGH were quite high (up to 730 ng/ml) but none of the animals showed increased growth rates (Hammer et al., 1985c).

The production of transgenic rabbits bearing copies of a hMT-IIA/ human GH gene construct has also been reported (Hammer et al., 1985a). In this instance 29 % of the animals born were transgenic, and of these 25 % were found to express HGH mRNA in their liver tissue, and 25 % only in their testicular tissue. No HGH mRNA could be detected in the livers of these latter animals even when induced by acute cadmium treatment. None of these animals grew at increased rates.

In summary, transgenic mice expressing either human, bovine or rat GH, or human GHRF under the control of either mouse or human metallothionein or murine MHC promoters have been shown to grow at vastly increased rates, up to twice that of their non-transgenic littermates. Both of the metallothionein promoters show enhanced activity when induced with heavy metals, and the human hMT-IIA promoter can also be induced with glucocorticoids. Transgenic rabbits, pigs, and one transgenic sheep have also been produced. Only a small proportion of the animals born were transgenic, somewhere in the order of 10 %. Of the transgenic rabbits, 25 % of those with MT-I/ HGH or hMT-IIA/ HGH constructs expressed the gene in their livers, and 25 % of those with the hMT-IIA/HGH construct expressed the gene in testicular tissue only. Approximately 60 % of the transgenic pigs produced contained detectable levels of HGH in their serum, but the site of gene expression is unknown. No transgenic animals, other than mice have been produced which

possessed increased growth characteristics. For the transgenic pigs at least, this may be due to the low growth promoting effect of HGH on pigs (Baile *et al.*, 1983).

1.5 Summary and aims of project

This introduction has described experiments which demonstrated the ability of each of the three hormones in the growth hormone cascade to stimulate growth, either by the injection of purified or synthesized hormone, by expression in transgenic animals, or both. The aim of the work described in this thesis, was to utilize the techniques for production of eukaryotic proteins in bacterial cells and for the controlled expression of hybrid genes in transgenic animals, in an attempt to improve the growth rates of farm animals.

Of the three most common farm animals, sheep, pigs and cattle, pigs were chosen for initial experiments. This was due to the ability of the pig to carry and bear multiple offspring (greater than ten), its high fertility rate, non-seasonal breeding, and reasonable gestation period (approximately 100 days).

The gene chosen for both expression in bacterial cells and in transgenic animals was the porcine GH gene. At the time when these experiments were initiated GH was the only one of the GH cascade hormones whose ability to stimulate growth had been demonstrated both by direct injection, and by expression in transgenic animals. Growth hormone is still the only hormone whose action on farm animals has been clearly demonstrated. The growth potentiating effects of GHRF and IGF-I have to date only been demonstrated in laboratory rats and mice under strictly controlled conditions. As the animal chosen for initial experiments was the pig, it was decided to use the homologous hormone in the hope that it would be more efficient in

promoting growth.

The initial aim of the project was therefore to isolate and characterize full length PGH cDNA and genomic clones (Chapter 2). These clones were then used to construct plasmids capable of expressing high levels of PGH in *E.coli* cells (Chapter 3), and as part of an expression plasmid capable of directing the production of PGH in transgenic animals (Chapter 4).

CHAPTER 2

THE ISOLATION AND CHARACTERIZATION OF PGH cDNA AND GENOMIC GENES

2.1 Introduction

The initial aim of the experiments described in this Chapter was to isolate a full length PGH cDNA clone for use in both bacterial and transgenic animal expression systems. Although no full-length PGH cDNA clones were available for use, the nucleotide sequence of the region of a PGH cDNA corresponding to the mature hormone was known (Seeburg *et al.*, 1983) thus enabling the generation of a suitable oligonucleotide hybridization probe. Subsequently, a number of papers appeared which indicated the involvement of sequences downstream from the poly A tail addition site in the polyadenylation of mRNAs (Woychik *et al.*, 1984; Gil and Proudfoot, 1984; McLauchlan *et al.*, 1985). The PGH genomic gene was therefore isolated from a porcine cosmid library, so as to provide these sequences for the transgenic animal constructs and also to enable a study of the PGH gene structure.

This Chapter details the construction and screening of a porcine pituitary cDNA library and the isolation and sequencing of PGH cDNAs, the screening of a porcine cosmid library, and the isolation and characterization of the genomic PGH gene. The analysis of the genomic gene by Southern blotting is also described.

2.2 Construction and screening of a porcine pituitary cDNA library

2.2.1 Construction of a cDNA library

A cDNA library of approximately 4000 individual recombinants was constructed using the plasmid vector pUC19 (Norrander *et al.*, 1983) as described in detail in Chapter 6 (6.3.3). Briefly, RNA was isolated from porcine pituitaries and poly A^+ RNA purified by fractionation on an oligo-dT cellulose column (6.3.3.i). This poly A^+ RNA was converted to double-stranded cDNA by the RNase H method of Gubler and Hoffman (1983; 6.3.3.ii)

and cloned into pUC19 by the addition of 8 base synthetic *EcoRI* linkers (6.3.3.iv). Following linkering, the cDNA was size fractionated through a low melting temperature agarose gel (6.3.2.ii) and the linkered cDNA of between 300 and 1500 bp recovered and ligated to *EcoRI* digested, dephosphorylated, pUC19. Transformation (6.3.3.v) into *E.coli* strain MC1061 (6.2.4) resulted in the formation of approximately 5000 ampicillin resistant colonies.

Small scale plasmid isolations (6.3.4.ii) were performed on ten of these transformants to determine what proportion of clones contained *EcoRI* inserts. Of the ten colonies chosen seven contained plasmids with *EcoRI* inserts ranging in size from 300 to 1000 bp (data not shown).

2.2.2 Screening for PGH cDNA clones

Clones containing PGH cDNAs were identified by screening colonies with a 27 base long synthetic oligonucleotide, GH.27 (6.2.5), which is complementary to the DNA sequence of Seeburg *et al.* (1983) coding for PGH amino acid residues 121 to 129.

Ampicillin resistant colonies were picked in duplicate onto nitrocellulose discs and processed using the method of Grunstein and Hogness (1975; 6.3.4.i). The GH.27 oligonucleotide was then end-labelled with ^{32}P and hybridized to the discs as described elsewhere (6.3.8.iii). Of the 400 colonies screened, nine hybridized to the GH.27 probe in duplicate, indicating that approximately 2 % of the transformants contain PGH cDNA sequences. An autoradiograph illustrating a number of the positives is shown in Figure 2-1. The nine positives were numbered from pPG.1 to pPG.9 in the order of their detection.

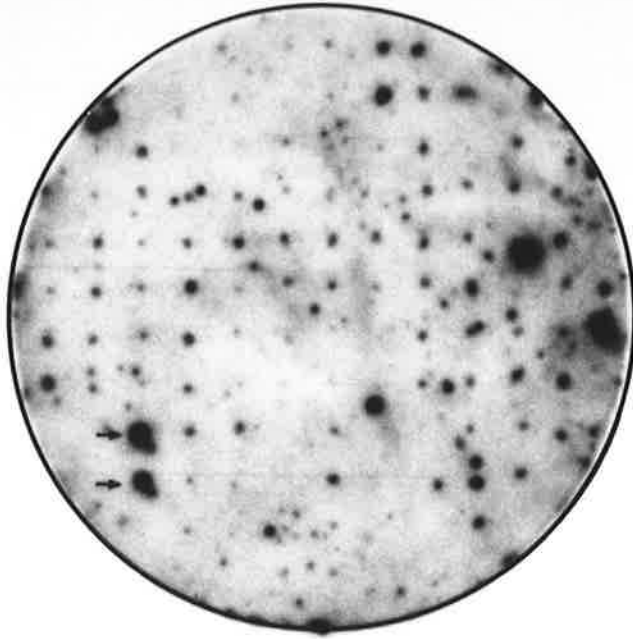
The identity of the positives and the size of their inserts was then determined by Southern blotting. Small scale plasmid preparations were performed (6.3.4.ii) for each of the positives and a 300 ng sample of each

FIGURE 2-1

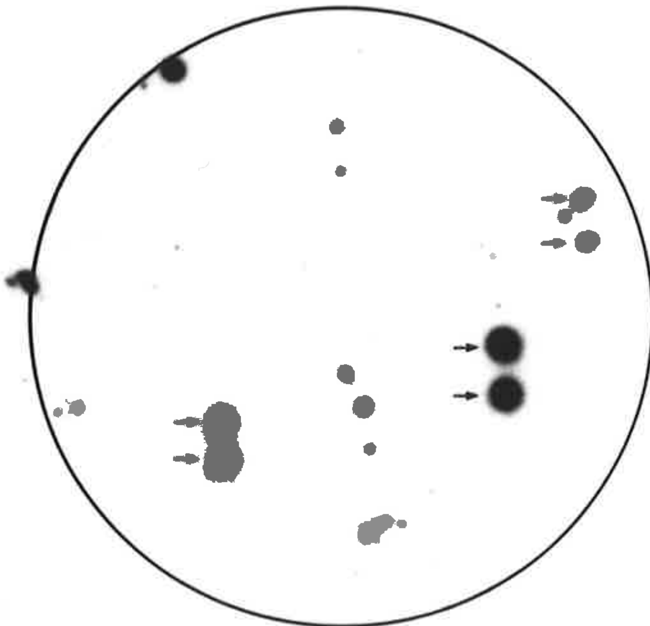
Screening for PGH cDNA clones

Nitrocellulose filters each containing approximately 50 duplicate colonies were processed (6.3.4.i) and hybridized (6.3.8.iii) to ^{32}P-kinased oligonucleotide, GH.27 (6.2.5). Following hybridization the discs were washed in 6 X SSC/0.1 % SDS at 65 ^{o}C for one hour, then in 2 X SSC/0.1 % SDS at 65 ^{o}C for one hour. Autoradiography of the washed discs revealed a number of colonies which hybridized to the synthetic probe in duplicate, and these have been marked with arrows.

A



B



digested with *Eco*RI (6.3.2.i), and electrophoresed on an agarose minigel (6.3.2.ii). Each of the plasmids contained *Eco*RI inserts (pPG.6 contained two inserts) which ranged in size from 400 to approximately 850 bp (Figure 2-2). This gel was processed and transferred to a nitrocellulose filter (6.3.2.iv) which was then hybridized to end-labelled oligonucleotide GH.27 (6.3.8).

Autoradiography of this filter revealed that all of the plasmids contained inserts which hybridized to the oligonucleotide probe (Figure 2-2), confirming that each of the clones contained PGH cDNA sequences. Only one of the two *Eco*RI inserts present in pPG.6 hybridized to the oligonucleotide, indicating that this plasmid probably arose due to a double cloning event.

2.2.3 Sequence analysis of PGH cDNA clones

The previous cloning of bovine cDNA clones indicated that the expected size of a full length PGH cDNA clone was approximately 800 bp (Miller *et al.*, 1980; Seeburg *et al.*, 1983). One cDNA clone of 800 bp, pPG.3, and two cDNA clones of greater than this size, pPG.5 and pPG.6, were therefore chosen for further study. The *Eco*RI insert of each of these three plasmids was purified (6.3.2.ii) and cloned into *Eco*RI digested M13 mp19 for sequence analysis by the dideoxy chain termination method of Sanger *et al.* (1977; 6.3.7). Each of the three plasmids contained sequences at their 5' end homologous to the 5' untranslated region of the bovine GH cDNA sequence of Seeburg *et al.* (1983). Each plasmid also possessed a poly A tail at the 3' end of its insert, ranging in length from 14 bp in pPG.3 to greater than 50 bp in both pPG.5 and pPG.6 (the exact length could not be determined due to sequence distortion by the poly A tail), explaining why clones of greater than the expected full length were obtained. As plasmid pPG.3 contained a long 5' untranslated region, and a poly A tail of only 14 bp it was

FIGURE 2-2

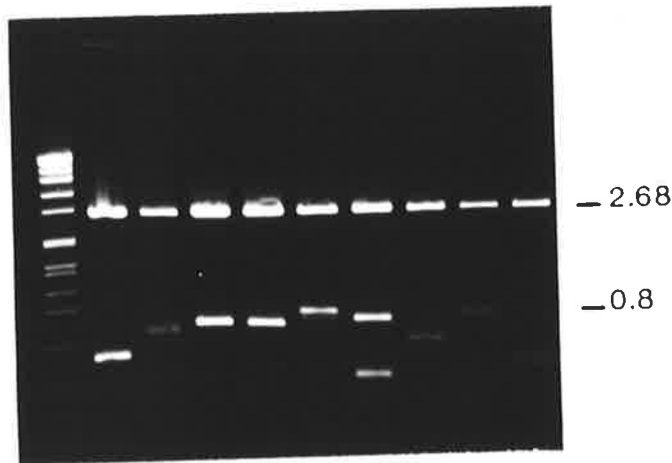
Analysis of oligonucleotide positive colonies by Southern blotting

A: Agarose gel illustrating *EcoRI* digests of each of the oligonucleotide GH.27 positive cDNA clones (2.2.2). The molecular weight markers are *EcoRI* digested bacteriophage SPP-1 DNA.

B: Southern blot of putative PGH cDNA clones probed with oligonucleotide GH.27. The agarose gel illustrated in A was processed and transferred to a nitrocellulose filter (6.3.2.iv). This filter was hybridized to end-labelled oligonucleotide GH.27 (6.3.8.iii), followed by washing in 2 X SSC/0.1 % SDS at 65 ^{o}C (2.2.2).

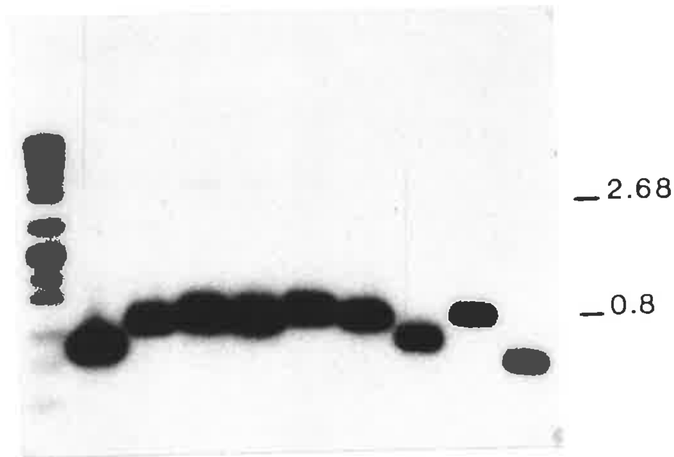
A

M 1 2 3 4 5 6 7 8 9



B

M 1 2 3 4 5 6 7 8 9



chosen for more detailed study by sequence analysis.

Sequence analysis of pPG.3

The entire sequence of the *EcoRI* insert of pPG.3 was determined by subcloning six different restriction fragments into M13 vectors, as illustrated in Figure 2-3, followed by sequencing using the chain termination method (6.3.7). The presence and position of each of these restriction sites was deduced from the sequence of the partial length PGH cDNA clone of Seeburg *et al.* (1983). By analysing the reaction products of each clone on both long and short 6% acrylamide/ 8M urea gels the entire sequence was determined in both orientations, and is shown in completed form in Figure 2-4.

The insert contains a 5' untranslated region of 41 bp in length, an open reading frame coding for pre-PGH of 648 bp, a 3' untranslated region of 102 bp and a poly A tail of 14 bp, flanked by the synthetic *EcoRI* linkers added *in vitro*.

2.3 Southern analysis of the PGH genomic gene

The organization of the genomic gene encoding PGH was studied by Southern blotting. Porcine genomic DNA (4 ug) isolated from tail tissue (6.3.1.ii) was digested with the restriction enzymes *BamHI*, *EcoRI*, *HindIII* and *NcoI* then electrophoresed through an agarose gel and transferred to a nylon membrane by alkaline blotting (6.3.2.iv). This membrane was then hybridized to the *EcoRI* insert of pPG.3 which had been radioactively labelled by nick-translation (6.3.5.i). After washing the membrane at a stringency of 0.1 X SSC /0.1% SDS at 65°C it was subjected to autoradiography (Figure 2-5). Bands were clearly visible in each of the tracks, except the *NcoI* digest which failed to precipitate properly and thus contained much less than 4 ug of DNA. Each digest produced a single intense band, and in

FIGURE 2-3

Sequencing strategy of cDNA clone pPG.3

The cDNA insert of plasmid pPG.3 was completely sequenced in both directions (2.2.3). Sequence was determined from EcoRI, SmaI/EcoRI and RsaI/EcoRI restriction fragments subcloned into M13 vectors mp18 and mp19. Sequence was generated by the dideoxy procedure and analysed on 6 % acrylamide/8 M urea gels (6.3.7).

100 bp

EcoRI

RsaI

SmaI

EcoRI

FIGURE 2-4

Nucleotide sequence of PGH cDNA clone pPG.3

The nucleotide sequence of the pPG.3 insert was assembled from the data generated as described in Figure 2-3. The sequenced insert contains 41 bp of 5' untranslated sequences, a 648 bp pre-PGH open reading frame, 102 bp of 3' untranslated sequence and is flanked by the synthetic EcoRI linkers added *in vitro* (2.2.3). The internal restriction enzyme sites which were used in generating the sequence data, and for subcloning in later Chapters, are indicated above the sequence. This sequence differs in two positions from the truncated PGH cDNA described by Seeburg *et al.* (1983). These differences are a conservative substitution at base number 507 and the alteration of a short region in the 3' untranslated sequences. Both of these alterations are indicated above the sequence of pPG.3 at the relevant positions. The first amino acid of the mature PGH molecule, a phenylalanine, has been underlined (residue number 27).

```
                                                        PstI
                                                 met ala ala gly pro arg thr
GAATTCCCTCAGACCACTCAGGGACCTGTGGACAGCTCACCGGCTGTG ATG GCT GCA GGC CCT CGG ACC
        10        20        30        40         50          60

ser val leu leu ala phe ala leu leu cys leu pro trp thr gln glu val gly ala
TCC GTG CTC CTG GCT TTC GCC CTG CTC TGC CTG CCC TGG ACT CAG GAG GTG GGA GCC
          80         90         100        110        120

phe pro ala met pro leu ser ser leu phe ala asn ala val leu arg ala gln his
TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT GCC AAC GCC GTG CTC CGG GCC CAG CAC
  130        140        150         160        170        180

leu his gln leu ala ala asp thr tyr lys glu phe glu arg ala tyr ile pro glu
CTG CAC CAA CTG GCT GCC GAC ACC TAC AAG GAG TTT GAG CGC GCC TAC ATC CCG GAG
     190        200        210         220        230         240
         RsaI
gly gln arg tyr ser ile gln asn ala gln ala ala phe cys phe ser glu thr ile
GGA CAG AGG TAC TCC ATC CAG AAC GCC CAG GCT GCC TTC TGC TTC TCG GAG ACC ATC
        250        260        270         280        290

pro ala pro thr gly lys asp glu ala gln gln arg ser asp val glu leu leu arg
CCG GCC CCC ACG GGC AAG GAC GAG GCC CAG CAG AGA TCG GAC GTG GAG CTG CTG CGC
300        310        320        330         340        350

phe ser leu leu leu ile gln ser trp leu gly pro val gln phe leu ser arg val
TTC TCG CTG CTG CTC ATC CAG TCG TGG CTC GGG CCC GTG CAG TTC CTC AGC AGG GTC
   360        370        380        390         400        410

phe thr asn ser leu val phe gly thr ser asp arg val tyr glu lys leu lys asp
TTC ACC AAC AGC CTG GTG TTT GGC ACC TCA GAC CGC GTC TAC GAG AAG CTG AAG GAC
       420        430        440        450         460
                                                 A            SmaI
leu glu glu gly ile gln ala leu met arg glu leu glu asp gly ser pro arg ala
CTG GAG GAG GGC ATC CAG GCC CTG ATG CGG GAG CTG GAG GAT GGC AGC CCC CGG GCA
          480        490        500        510         520

gly gln ile leu lys gln thr tyr asp lys phe asp thr asn leu arg ser asp asp
GGA CAG ATC CTC AAG CAA ACC TAC GAC AAA TTT GAC ACA AAC TTG CGC AGT GAT GAC
  530        540        550         560        570         580

ala leu leu lys asn tyr gly leu leu ser cys phe lys lys asp leu his lys ala
GCG CTG CTT AAG AAC TAC GGG CTG CTC TCC TGC TTC AAG AAG GAC CTG CAC AAG GCT
     590        600        610        620        630

glu thr tyr leu arg val met lys cys arg arg phe val glu ser ser cys ala phe
GAG ACA TAC CTG CGG GTC ATG AAG TGT CGC CGC TTC GTG GAG AGC AGC TGT GCC TTC
          650        660        670        680        690
                                         CTGGTG
***                                       \  /
TAG TTGCTGGGCATCTCTGTTGCCCCTCCCCAGTACCTCCCTTGACCCTGGAAAGTGCCACCCCAATGCCTGCT
  700       710       720       730       740       750       760       770

GTCCTTTCCTAATAAAACCAGGTTGCATCGTAAAAAAAAAAAAGGAATTC
     780       790       800       810       820
```

FIGURE 2-5

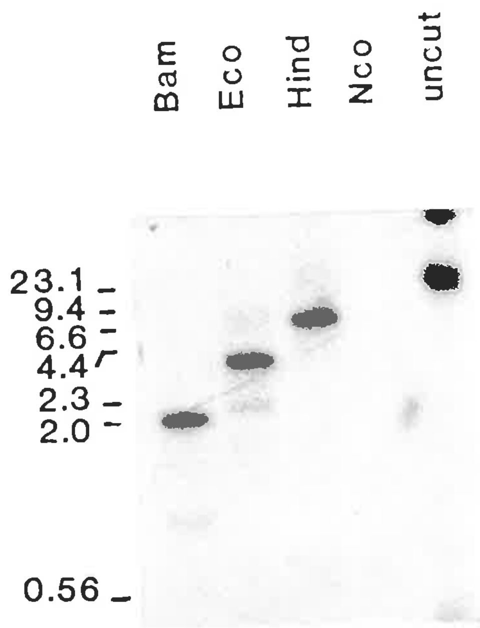
Genomic blot of pig DNA probed with pPG.3

Restriction enzyme digested samples of pig DNA (4 ug) isolated from tail tissue (6.3.1.ii) were fractionated by agarose gel electrophoresis, transferred to Zeta-probe membrane (6.3.2.iv), and hybridized to plasmid pPG.3 which had been radioactively labelled by nick-translation to a specific activity of 3×10^{8} cpm/ug (2.3). Following hybridization and washing at high stringency (6.3.2.iv), autoradiography of the membrane was performed. The presented autoradiograph demonstrates the presence of pPG.3 homologous sequences in all tracks, except the NcoI track, which failed to precipitate properly and thus contained much less than 4 ug of DNA. The size of the bands (in kb) are as follows:

BamHI - 2.1, 1.0

EcoRI - 3.8, 2.3

HindIII - 9.4


Bam
Eco
Hind
Nco
uncut
23.1
9.4
6.6
4.4
2.3
2.0
0.56

the case of BamHI and EcoRI digests, a second fainter band, a result consistent with the GH gene existing as a single copy sequence in the porcine genome. The size of each band is described in the legend to Figure 2-5.

2.4 Cloning of the PGH genomic gene

2.4.1 Screening of a porcine cosmid library

The genomic gene was isolated from a porcine cosmid library previously constructed by P.Haley and kindly made available by R.Crawford (Howard Florey Institute, Melbourne). Duplicate nitrocellulose discs , which had previously been used by the Melbourne group to screen the library were supplied. These discs were screened for PGH gene sequences by hybridization to the EcoRI insert of the PGH cDNA containing plasmid pPG.3 (2.2.3), which had been radioactively labelled by nick-translation (6.3.5.i). After washing the discs at a stringency of 0.2 X SSC /0.1% SDS at 65 ^{o}C and autoradiography, only a single clear positive was present in duplicate (Figure 2-6). As the exact number of colonies screened per disc, or the complexity of the library were not known, the expected frequency of positives could not be determined.

Colonies in the region surrounding the positive were picked (kindly done by C.Troiani, Howard Florey Institute, Melbourne) into L-broth plus ampicillin and various dilutions of the bacterial suspension were plated out onto L-broth plus ampicillin plates and grown overnight. Approximately 400 individual colonies which arose from these platings were picked in duplicate onto nitrocellulose filters and following overnight growth, processed for screening as described (6.3.4.i). The filters were screened by hybridization to the nick-translated EcoRI insert of pPG.3, and washed in 0.2 X SSC / 0.1% SDS at 65 ^{o}C. Autoradiography of the washed discs revealed

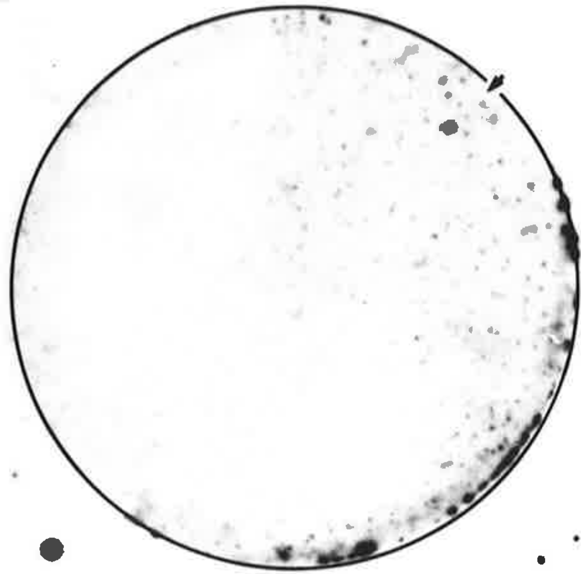
FIGURE 2-6

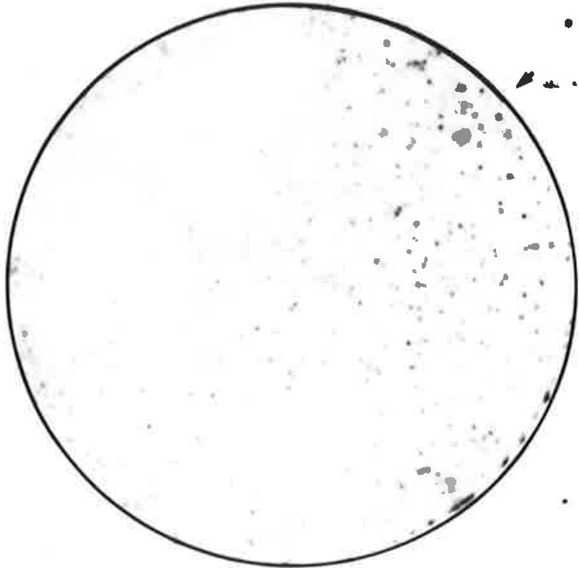
Screening cosmid clones for PGH sequences

A: First round screening of a porcine cosmid library. Duplicate discs containing the only detected first round positive (2.4.1), which is indicated with an arrow, are illustrated. The discs were screened with the nick-translated *Eco*RI insert of the PGH cDNA plasmid, pPG.3 (2.2.3). Wash conditions were 0.2 X SSC/0.1 % SDS at 65 ^{o}C.

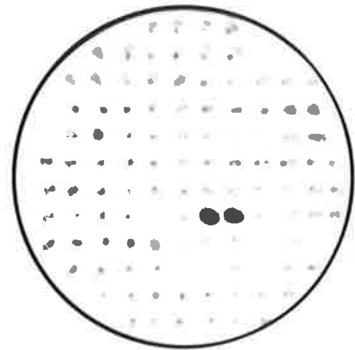
B: Second round screening of porcine cosmids. Colonies surrounding the region of the positive detected in first round screening were picked in duplicate onto a nitrocellulose filter and screened for PGH sequences by hybridization to the nick-translated insert of pPG.3. Wash conditions were 0.2 X SSC/0.1 % SDS at 65 ^{o}C. The single positive detected was named cPGH.1 (2.4.1).

A





B



a single clone which hybridized to the pPG.3 probe in duplicate (Figure 2-6). This clone, cPGH.1, was picked and grown on a large scale for cosmid purification (6.3.1.i).

2.4.2 Analysis of cosmid clone cPGH.1

The cosmid clone cPGH.1 was initially characterized by Southern blotting. This allowed the positive identification of PGH gene sequences and enabled the generation of a PGH gene restriction map. This analysis also allowed fragment identification for subsequent subcloning and sequence analysis.

Cosmid DNA was digested with a number of different restriction enzymes, both in single and double digests, then electrophoresed through agarose gels and transferred to nitrocellulose filters (6.3.2.iv). These filters were then hybridized to the nick-translated insert of pPG.3. Following washing in 0.1 X SSC /0.1% SDS at 65 ^{o}C the filters were then subjected to autoradiography (Figure 2-7).

Examination of the resulting autoradiographs indicated that even at high stringency, the cosmid DNA hybridized strongly to the PGH cDNA probe. The previous sequencing of the PGH cDNA clone pPG.3 identified the location of unique PstI and SmaI restriction sites within the coding region (Figure 2-4). Assuming that the cosmid contained PGH gene sequences, it was expected that these two sites would be conserved. As the cosmid Southerns presented in Figure 2-7 illustrate, there is an internal SmaI site within the region homologous to the PGH cDNA probe, resulting in the generation of two PGH cDNA positive bands. Digestion with PstI produces a single band of 1.6 kb which binds the cDNA probe. As the PstI site is at the very 5' end of the coding region (Figure 2-4) it is entirely consistent that only a single band is detected, with the second PstI site occurring close to the 3' end of the PGH gene. By utilizing the known location of the SmaI and PstI sites

FIGURE 2-7

<u>Southern analysis of cosmid clone cPGH.1</u>

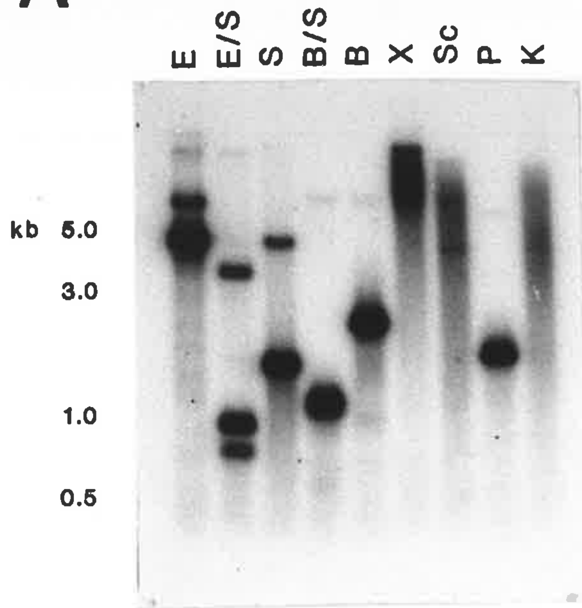
A and B: Southern analysis of cPGH.1 DNA (2.4.2). Southern blots containing cosmid DNA digested with a range of restriction enzymes were hybridized to the nick-translated insert of the PGH cDNA clone, pPG.3 (2.2.3). The sizes of the bands (in kb) detected in these Southerns were as follows:

A:
- EcoRI- 5.9, 4.0
- SmaI - 3.95, 1.6
- BamHI- 2.0
- EcoRI/SmaI- 3.1, 0.82, 0.62
- BamHI/SmaI- 1.0, 0.98
- PstI - 1.6

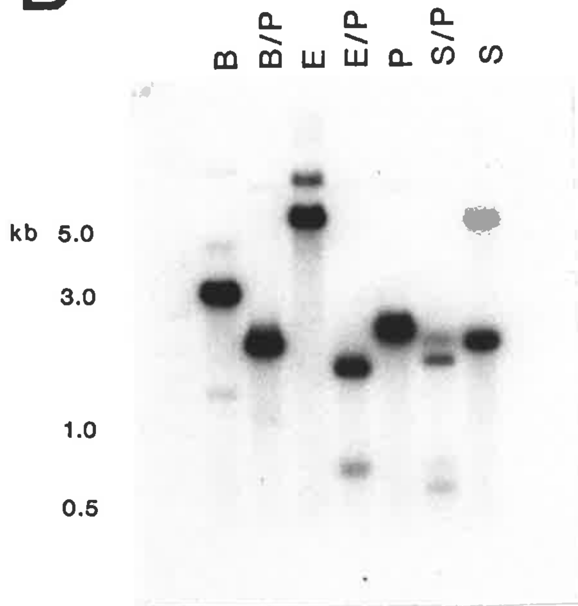
B:
- BamHI- 2.1, 1.0
- EcoRI- 6.0, 4.1
- PstI - 1.7
- SmaI- 4.0, 1.6
- BamHI/PstI- 1.7, 0.9
- EcoRI/PstI- 1.3, 0.5
- SmaI/PstI - 1.35, 0.53, 0.4

C: The known location of unique SmaI and PstI sites in the PGH cDNA sequence (Figure 2-4) and the double digest data shown above was used to construct a restriction map of the genomic PGH gene. The location of each of these restriction sites was later confirmed by nucleotide sequencing. The identity of the two cPGH.1 fragments which were isolated and subcloned into pUC19 to generate pGHS.9 and pGHB.3 (2.4.2) is also indicated.

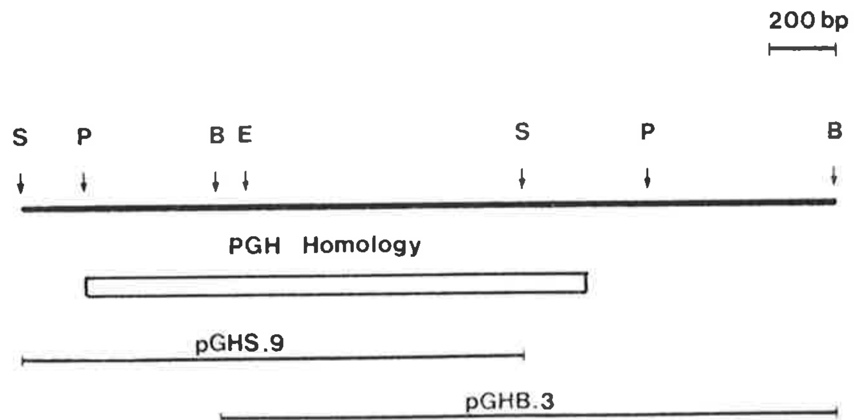
Key to enzymes:
- E - EcoRI
- S - SmaI
- B - BamHI
- X - XhoI
- Sc- SacI
- P - PstI
- K - KpnI



A
E
E/S
S
B/S
B
X
Sc
P
K
kb 5.0
3.0
1.0
0.5



B
B
B/P
E
E/P
P
S/P
S
kb 5.0
3.0
1.0
0.5



C
200 bp
S
P
B E
S
P
B
PGH Homology
pGHS.9
pGHB.3

and the double digest data, a restriction map of the PGH gene area could be constructed (Figure 2-7).

2.4.3 Sequencing the PGH gene contained in cosmid cPGH.1

The restriction map generated from the Southern blot data indicated that the entire gene was not present on any single BamHI, EcoRI, PstI or SmaI restriction fragment (the single PstI fragment does not contain 5' untranslated or promoter sequences, Figure 2-4). Two subclones were therefore constructed which between them contained the entire gene. Cosmid DNA was digested with either BamHI or SmaI and fractionated on a low melting temperature agarose gel (6.3.2.ii) and the 2.0 kb BamHI and 1.6 kb SmaI bands which had been shown to hybridize to the PGH cDNA were purified and individually subcloned into pUC19 (6.3.4.iii). These plasmids were named pGHB.3 (BamHI) and pGHS.9 (SmaI). The 1.6 kb SmaI band was also cloned into M13 mp19 and subjected to sequence analysis to confirm that the cosmid contained PGH gene sequences. This sequence was found to be identical to that adjacent to the SmaI site of the cDNA sequence (see below).

The strategy used to determine the nucleotide sequence of the 2 kb region containing the PGH gene is illustrated in Figure 2-8. Most of the data was generated by subcloning specific fragments from cosmid subclones pGHB.3 and pGHS.9 into mp18 or mp19 vectors. In addition, a number of BAL-31 (6.3.6.ii) and randomly generated clones were analysed to fill in the regions which contained no convenient restriction sites. The random clones were generated by partially digesting the purified insert from plasmid pGHB.3 with the four base cutting enzyme HaeIII, and selecting and subcloning partial digest products of approximately 1 kb in length into SmaI digested mp19.

The analysis of the clones described above enabled the determination of approximately 80% of the final sequence. The sequence of regions not

FIGURE 2-8

Sequencing of the PGH gene contained in cosmid cPGH.1

Subclones from plasmids pGHS.9 and pGHB.3 were generated in M13 vectors by a variety of different methods (2.4.3). A number of clones were generated using restriction enzymes and BAL-31 exonuclease (6.3.6.ii). The region and orientation of DNA sequence covered by each of these clones is indicated on the restriction map by arrows. Sequence generated by priming sequencing reactions with PGH-specific oligonucleotides Seq.1 to Seq.4 (6.2.5) is indicated by arrows beginning with a square. Solid lines indicate exon regions, and thin lines, untranslated sequences.

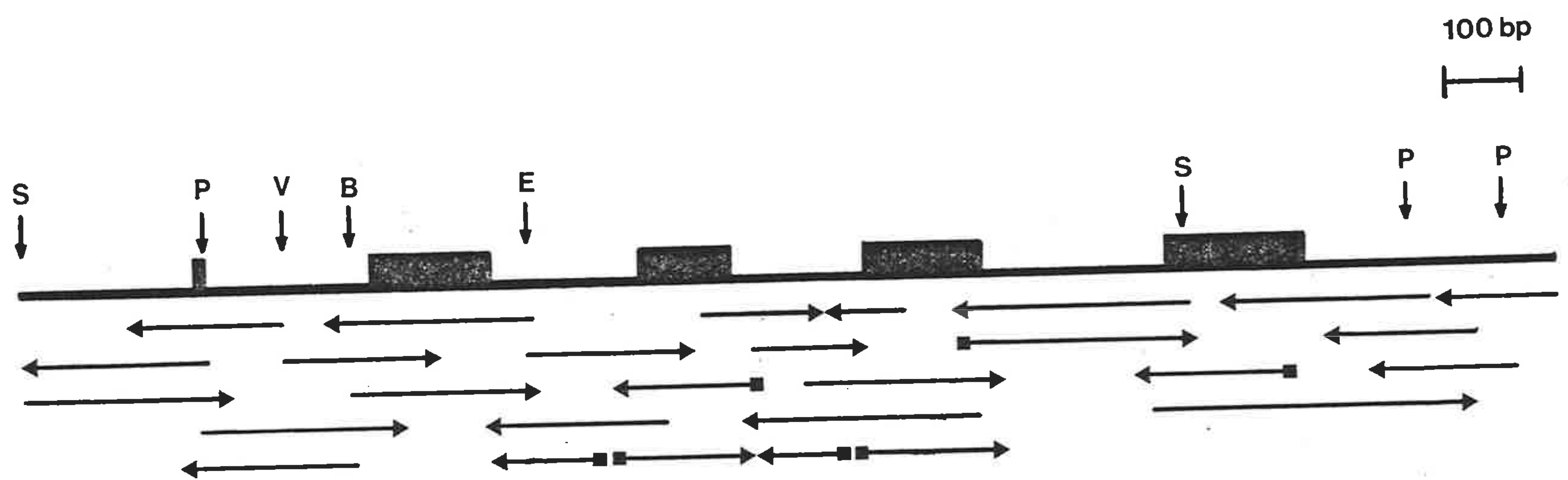
S - SmaI

P - PstI

V - EcoRV

B - BamHI

E - EcoRI


100 bp
S
P
V
B
E
S
P
P

determined from restriction fragment clones was generated by synthesizing a number of short oligonucleotides (Seq.1 to Seq.4; 6.2.5) and using these to specifically prime sequencing reactions at the desired positions.

The final sequence of the PGH gene is presented in Figure 2-9. As Figure 2-8 illustrates, the promoter, exon and intron regions were completely sequenced in both orientations. The only region determined in only one orientation was the very last 100 bp of the 3' untranslated region. Each base in this region was however determined from two independent clones.

The complete 2231 bp long nucleotide sequence of the PGH gene incorporates 238 bp of sequence 5' to the ATG, the entire coding region which is interrupted by four introns, and 414 bp of 3' sequence extending 312 bp beyond the polyadenylation site.

2.5 Discussion

2.5.1 PGH cDNA sequences

The construction of a porcine pituitary cDNA library in pUC19 allowed the isolation of PGH cDNA clones using a synthetic DNA probe. One of the cDNA clones isolated, pPG.3, which was found to contain an insert of the expected full length, was completely sequenced.

The open reading frame of the pPG.3 cDNA insert was found to code for a 216 amino acid pre-hormone, identical in amino acid sequence to the partial length PGH cDNA clone of Seeburg et al. (1983). This clone provides for the first time the complete sequence of the region encoding the PGH signal sequence and 41 bases of the 5' untranslated region.

The nucleotide sequence within the coding region is very highly conserved, with only a single base difference from the sequence of Seeburg et al. (1983). This altered base is a conservative substitution at base 501

FIGURE 2-9

Nucleotide sequence of the PGH genomic gene

The complete 2231 bp sequence is illustrated (2.4.3). The open reading frame which encodes pre-PGH is indicated, as are bases which differ in sequence from the previously studied PGH cDNA sequence, pPG.3 (Figure 2-4). Base changes which result in amino acid substitutions are marked with an asterisk. The single base in the 3' untranslated region which differs from pPG.3 is also indicated below the sequence. The location of the cap site (+1) was inferred from the position of the rat and human GH gene cap sites (Page *et al.*, 1981; DeNoto *et al.*, 1981).

Putative promoter and polyadenylation sequences, such as the TATA, AATAAA and GT-rich sequences are underlined, and the position at which the poly A tail is added is indicated with an arrow. The variant GC donor splice site is located in the first intron, around base +72.

The *SmaI* restriction site utilized for subcloning in latter Chapters is indicated.

```
CCCGGGGGACATGACCCCAGAGGAGGAGCGGGAACAGGATGAGTGGGAGGAGGTTCTAAATTATCCATTAGCACATGCCTGCCAGTGGGCCATGCATAAATGTATAG
                              -150                                                          -100

AGAAAATAGGTGGGGGCAGAGGGAGAGAGAAGAGGCCAGGG TATAAAA AGGGCCCAAAAGGGACCAATTCC A GAATCCCAGGACCCAGCTCCCCAGACCACTC
                         -50                                                  +1

                                        met ala ala g
AGGGACCTGTGGACAGCTCACCGGCTGTG ATG GCT GCA G GCAAGTGCCCCTAAAATCCCAGTGGCTTGGTGTGTTCTGAAGGGTGACGTGGGGGCCATGCAG
                     +50                                                         +100

ATGGATGGGGCACCAACCTTGGGCTTTGGGGTTTCCGAATGTGAGCATGGATATCTACTCCTAGATATGAGGCCAAGTTTAAATGTCCCTGGGGGAGGGGGAGGAG
              +150                                                   +200

                                                                          ly pro arg thr ser ala leu leu ala
AAGGGACAGGGCTGGTGGAGCCAGGCCTCTTGTCTCTGGGATCCCTCTCTCACGGGCCCTCCTGGTCTCTAG GC CCT CGG ACC TCC GCG CTC CTG GCT
    +250                                                  +300                                 T*

phe ala leu leu cys leu pro trp thr arg glu val gly ala phe pro ala met pro leu ser ser leu phe ala asn ala
TTC GCC CTG CTC TGC CTG CCC TGG ACT CGG GAG GTG GGC GCC TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT GCC AAC GCC
        +350                            A*          A                          +400

val leu arg ala gln his leu his gln leu ala ala asp thr tyr lys glu phe
GTG CTC CGG GCC CAG CAC CTG CAC CAA CTG GCT GCT GAC ACC TAC AAG GAG TTT GTAAGCTCCCCAGGGAGGGTGCTGGAGGGGGGTGG
                                    +450        C                                           +500

TGGAGAGGGGTGAATTCGTCCCTCTCTGCCTAGTGGGAGGAAAATGAGGGGTTCTGGAGTATTGAGGCCAACCGAAGATGCTATCAGGTGAGTGTAAACTGAAGGGG
                                        +550                                                +600

                                                                       glu arg ala tyr ile pro glu gly gln
ATTCCCAAGAAAAGCAGCAAGGAGAACCGCGCCCCAGTGTAGACCTGGATGGCTGTCCCCTCTCCCAG   GAG CGC GCC TAC ATC CCG GAG GGA CAG
                                 +650                                                      +700

arg tyr ser ile gln asn ala gln ala ala phe cys phe ser glu thr ile pro ala pro thr gly lys asp glu ala gln
AGG TAC TCC ATC CAG AAC GCC CAG GCT GCC TTC TGC TTC TCG GAG ACC ATC CCG GCC CCC ACG GGC AAG GAC GAG GCC CAG
                                                +750

gln arg ser
CAG AGA TCG GTGAGTGGCCACCTGCCACCTGCCAGCCGGGGAGCAGGGGCCTCCCTCTTCCTAAGAAGGCTGCCCCATCTCTCATCATCAGGGCCTTGGGCGGC
      +800                                               +850

                                                                                                        asp
CTTCTCCCCGAGCTGGTGGGGGTGATGGTGGCAGAGGGCGGGGTGGTGAGGGGGACGCCCACCGGCGGAGGCAGCGCCCCCCATCCACGCATCTGCCCGCAG GAC
+900                                          +950                                                    +1000

val glu leu leu arg phe ser leu leu leu ile gln ser trp leu gly pro val gln phe leu ser arg val phe thr asn
GTG GAG CTG CTG CGC TTC TCG CTG CTG CTC ATC CAG TCG TGG CTC GGG CCC GTG CAG TTC CTC AGC AGG GTC TTC ACC AAC
                                                            +1050

ser leu val phe gly thr ser asp arg val tyr glu lys leu lys asp leu glu glu gly ile gln ala leu met arg
AGC CTG GTG TTT GGC ACC TCA GAC CGC GTC TAC GAG AAG CTG AAG GAC CTG GAG GAG GGC ATC CAG GCC CTG ATG CGG  GT
                +1100                                                              +1150

GGGGAGGCGCGCTCGGGTCCCGCACACTGGGGCCCATGCCGGCTCTCTCCCGGCTGAGCGGAGCGGTGGGGGGACGCACGTGGGCTGGGGGAGAGGGTCCCGATGCT
                                   +1200                                                   +1250

CTCTCTGTAGCAGTTCACTCTCGACCCGGAGAAATCTTTTCCTCATTTCCCCCTGCGGAGTCTTCCCTCTTTGTCCTTCTCCAAGCATGGAGGGGAGGGTGGAAGAC
                             +1300                                                 +1350

                                                                                     SmaI
                                                                 glu leu glu asp gly ser pro arg ala gly gln
GGAGGGGACAGGAGAGCGCCGCTGCCAAGGACTCGGCCTCTGTCTCTCTCTCCCTTTTGCAG   GAG CTG GAG GAT GGC AGC CCC CGG GCA GGA CAG
                  +1400                                                      A

ile leu lys gln thr tyr asp lys phe asp thr asn leu arg ser asp asp ala leu leu lys asn tyr gly leu leu ser
ATC CTC AAG CAA ACC TAC GAC AAA TTT GAC ACA AAC TTG CGC AGT GAT GAC GCG CTG CTT AAG AAC TAC GGG CTG CTC TCC
                                    +1500                                                            +1550

cys phe lys lys asp leu his lys ala glu thr tyr leu arg val met lys cys arg arg phe val glu ser ser cys ala
TGC TTC AAG AAG GAC CTG CAC AAG GCT GAG ACA TAC CTG CGG GTC ATG AAG TGT CGC CGC TTC GTG GAG AGC AGC TGT GCC
                                                                  +1600

phe ***
TTC TAG TTGCTGGGCATCTCTGTTGCCCCTCCCCAGTACCTCCCCTGACCCTGGAAAGTGCCACCCCAATGCCTGCTGTCCTTTCCT AATAAA ACCAGGTTGC
             +1650                                T                     +1700

ATCGT ↓ ATTGTCTGAGTA GGTGTCACT CTGCGATGGAGGGAGGTGGGGCAGTAGGGCAAGGGGTGGGGGTGGGAAGACAACCTGCAGGCATCCTTGGGGGTCTC
           +1750                                                   +1800

CTGGGGACCTAGACACTGAATGATGGTTGACCCGGCTTCTTCCTGGGCTTGAAAGAGCAGGCACATTACCTTCTCTCTGTTACACACCCACTGCACCCACTGCTCAG
     +1850                                                  +1900

GTCTGCAGTCCAGCTTGCTGGGCACTCATAGGTCAGGACCACCCCCCATCCTGCTACACCCCCCGCCTCCCATAAAGTACCCAAGAATGGAAAGAGATGAAAGCAAG
                                                +2000                                                +2050
```

contained within the region homologous to the synthetic oligonucleotide used as the hybridization probe (Figure 2-4). There are also some slight discrepancies between the sequence of pPG.3 and the sequence of Seeburg *et al.* (1983) in the 3' untranslated region (Figure 2-4).

2.5.2 Southern analysis using a PGH cDNA hybridization probe

When the cDNA insert of pPG.3 was used to probe Southern blots of porcine genomic DNA digested with three different restriction enzymes, a single strongly hybridizing band was detected in each track. Enzymes *Bam*HI and *Eco*RI also produced a second, fainter band, indicating that if the gene was in fact present as a single copy, the genomic gene must contain internal sites for both of these enzymes, somewhere near the end of the region homologous to the cDNA (as was later discovered to be correct). The third enzyme used, *Hin*dIII, produced only a single clear band. Taken together these data indicated that only a single copy of the GH gene exists in the porcine genome (per haploid chromosome complement), a situation analogous to the bovine and rat genomes, but very different to that of humans (1.2.2.iii).

2.5.3 Isolation and analysis of the genomic PGH gene

The PGH cDNA clone pPG.3 (2.2), was used to screen a porcine cosmid library and isolate a clone containing PGH gene sequences (2.4). Southern analysis of the cosmid clone identified major *Bam*HI and *Eco*RI hybridizing bands which were equal in size to those detected in genomic Southerns utilizing the same hybridization probe (2.5.2; Figures 2-5, 2-7). Nucleotide sequencing of the gene contained within the cosmid revealed that the entire coding region was present (648 bp), along with 178 bp of the promoter region, 61 bp of 5' untranslated sequence, four introns of 242, 210, 197 and 278 bp respectively, and 414 bp of 3' non-coding sequence (Figure 2-9). The gene contains four base alterations within the coding region

relative to the previously sequenced PGH cDNA clone, pPG.3 (2.2.3). The base substitutions, which are illustrated in Figure 2-9, result in the alteration of two amino acid residues within the signal peptide. The remaining two differences are silent substitutions. There is also one base change in the 3' untranslated region relative to the pPG.3 cDNA sequence (Figure 2-9).

Sequence analysis of the PGH genomic gene revealed that one of the intron sequences contains a variant 5' donor splice site. The highly conserved GT dinucleotide which usually occurs at this position (Breathnach and Chambon, 1981) has been replaced with a GC dinucleotide. Similar GC donor sites have been described in a small number of other genes. In one instance (the murine alpha A-crystallin gene) the variant sequence was present at a rarely used alternative splice site (King and Piatigorsky, 1983). The other reported cases of GC splice sites occur in the chicken alpha D-globin (Dodgson and Engel, 1983), duck alpha D-globin (Erbil and Niessing, 1983), human Cu/Zn superoxide dismutase (SOD-1; Levanon _et al._, 1985) and murine adenine phosphoribosyltransferase (Dush _et al._, 1985) genes. The variant sequence in the SOD-1 gene is known to be functional as the GC sequence has been shown to be present in the first intron of both of the human alleles (Levanon _et al._, 1985). As the GH gene appears to exist as a single copy in the porcine genome (2.5.2) the GC splice site present in the sequenced PGH gene must be either functional, or allelic. As recent _in vitro_ splicing experiments indicate that GC splice sites are functional, albeit at a reduced efficiency relative to GT sequences (Aebi _et al._, 1986), the former possibility is likely to be correct.

Previous studies which have compared the promoter and 3' sequences of GH genes were performed using only limited data (Miller _et al._, 1980; Woychik _et al._, 1982; Miller and Eberhardt, 1983). Comparison of the newly

derived PGH gene sequence to the previously determined rat (RGH), human (HGH), and bovine (BGH) GH genes and the human placental lactogen (HPL) gene sequences was carried out to investigate if the additional data allowed any further insights into the sequences involved in GH gene expression or into GH gene evolution. As has previously been shown (Miller and Eberhardt, 1983) the HPL gene, which is expressed in different tissues to the GH gene, shares homology to the promoter region of GH genes, and was included in the comparison to indicate if any conserved sequences were GH specific.

The sequence comparison illustrated in Figure 2-10 indicates that the promoters of each of the studied sequences share a high degree of homology. Sequences conserved between GH promoters are also present within the HPL gene, indicating that they are probably not involved in GH tissue-specific expression, as was previously proposed by Woychik _et al_. (1982). This conclusion is consistent with recent studies on the RGH gene promoter which have indicated that a tissue-specific enhancer lies within this gene somewhere between nucleotides -235 and -181 (Nelson _et al_., 1986; 1.2.2.iv), and hence upstream from the region studied here. Therefore the only identifiable promoter element within the region of the PGH gene sequenced here is the "TATA" box present at around position -30 (Figure 2-9). Figure 2-10 indicates that the 5' untranslated regions of the GH and HPL genes are also conserved to a surprising degree.

Analysis of the 3' sequences of the PGH gene allowed the identification of sequences which have been shown to be important in the polyadenylation process. These include the AATAAA (Proudfoot and Brownlee, 1976) sequence located shortly before, and the GT rich sequence (McLauchlan _et al_., 1985) located shortly after the polyadenylation site (Figure 2-9).

The comparison of the 3' sequences of the PGH gene to all of the

FIGURE 2-10

Comparison of GH and HPL gene promoter and 5' untranslated sequences

Porcine (P), bovine (B), human (H) and rat (R) GH genes and the human placental lactogen (HPL) gene were aligned to determine the extent of sequence homology (2.5.3). Asterisks indicate homology between adjacent sequences. The PGH sequence is numbered with respect to the distance from the cap site (+1).

```
     5'                                       -150                                              -100
P     CCCGGGGGACATGAC----------------CCCAGAGGAGGAGCGGAACAGGATGAGTGGGAGGAGGTTCTAAATTATCCATTAGCACATGCCTGCCAGTGG
      ** ************                ******* **** **************** ***************************** ** *********
B    -CCTGGGGGACATGAC----------------CCCAGAGAAGGAACGGAACAGGATGAGTGAGAGGAGGTTCTAAATTATCCATTAGCACAGGC-TGCCAGTGG
         ***  * *****                **** *     *      *** *** *** ****** ********************** **  * ******
H    -GAAGGGAAAGATGAC-------------AAGCCAGGGGCATGATCC---CAGCATGTGTGGGAGGAGCTTCTAAATTATCCATTAGCACAAGCCCGTCAGTGG
          ****** * *              *   **** *     *     *   ****************************** *********  ********
R    -CGGTGGAAAGGTAAGATCAGGGACGTG-ACCGCAGGAGAGCAGTGGGGACGCGATGTGTGGGAGGAGCTTCTAAATTATCCATCAGCACAAGC-TGTCAGTGG
          ******   *              *   **** *     *     *   *****************************  *********  ********
HPL  -GAAGGGAAAGACGAC-------------AAGCCAGGGGCATGATCC---CAGCATGTGTGGGAGGAGCTTCTAAATTATCCACTAGCACAAGCCCGTCAGTGG

                                                      -50                                                  +1
P    ------GCCATGCATAAATGTATAGAGAAAA--TAGGTGGGGGCAGA---GGGAGAGAGA--AGAGGCCAGGGTATAAAAAGGGCCCAAAAGGGACCAATTCCA
            ** ***************** * *   ********* * *   **********  *** *************** ******  **************
B    ------TCCTTGCATAAATGTATAGAGCACA--CAGGTGGGGGGAAA---GGGAGAGAGAGAAGAAGCCAGGGTATAAAAATGGCCCAGCAGGGACCAATTCCA
            ** ************ * ** * *  ********** **    ********    **  *************** ******  ** ****   ** *
H    ---CC--CCATGCATAAATGTACACAGAA-A--CAGGTGGGGGCAACAGTGGGAGAGA----AGGGGCCAGGGTATAAAAAGGGCCCACAAGAGACCGGCTCAA
        **  ***** ********* *  **  *  **** *       *   ***         *  **  ****************   **** ****   ** *
R    -CTCCAGCCATGAATAAATGTATAGGGA-AAGGCAGGAG------CCTTGGGGTCGAGGAAAACAGGT-AGGGTATAAAAAGGGCATGCAAGGGACCAAGTCCA
            ***** ********* *  ** **  **** *           ***     **  ** **  ****************   **** ****   ** *
HPL  -----CCCCATGCATAAATGTACACAGA-AA--CAGGTGGGGTCAAGCA-GGGAGAGAGA--ACTGGCCAGGGTATAAAAAGGGCCCACAAGAGACCGGCTCTA

                                                                      3'
P    -GAATCCCAGG-ACCCAGCTCCCCAGACCACTCAGGGACCTGTGGACAGCTCACC-GGCTGTG ATG
      * ******** ****** ** ****** ******** *****************  *** **
B    -GGATCCCAGG-ACCCAGTTCACCAGACGACTCAGGGTCCTGTGGACAGCTCACC-AGCTATG ATG
      **********  ****  ** **  ** ****************** ******* ****
H    -GGATCCCAGG-GCCCAACTCCCCGAACCACTCAGGGTCCTGTGGAC-GCTCACCTAGCTGCA ATG
      * * ***  * *****  *  ** ***  ****** ********** * ****  **  **
R    -GCA-CCCTCGAGCCCAGATT-CCAAACTGCTCAGG-TCCTGTGGACAGATCACTGAGTGGCG ATG
      * * ***  * *****  *  ** ***  ****** ************ ****  ******
HPL  -GGATCCCAAG-GCCCAACTCCCCGAACCACTCAGGGTCCTGTGGACAGCTCACCTAGTGGCA ATG
```

available GH and HPL sequences has revealed that these are also conserved to a surprising extent, between both GH and HPL genes (Figure 2-11). This homology extends down to the polyadenylation site for all of the genes, and far beyond for each of the genes except for the RGH gene, which diverges rapidly beyond this point. Taken with the high conservation of promoter sequences analysed above (which are probably not involved in tissue-specific expression) and of 5' untranslated sequences, this high degree of conservation indicates that the sequences surrounding GH genes may be evolving at a very slow rate, due to some unknown evolutionary constraint. This conservation of sequence across species also occurs in GH and HPL gene intron sequences (data not shown). In the case of the HGH and HPL genes this conservation may be the result of the homogenization of multi-gene families which has been observed in a number of systems (see Dover and Flavell, 1984 for review). However as rat (Page _et al._, 1981), bovine (Woychik _et al._, 1982) and porcine (2.5.2) genomes appear to contain only single copies of the GH gene and no other closely related sequences, the mechanism of conservation in these animals is unknown. This indicates that if these sequences are not functional (which is quite possible), the evolution of GH genes may be governed by some mechanism capable of acting on single genes.

In summary, both cDNA and genomic copies of the PGH gene have been isolated and sequenced. The cDNA clone contains the entire coding region and is suitable for use in both bacterial and transgenic animal expression systems. The PGH gene isolated codes for an identical mature PGH protein, and contains the 3' sequences necessary for efficient polyadenylation. The cosmid gene was found to contain a variant donor splice site in the first intron.

FIGURE 2-11

Comparison of GH and HPL 3' sequences

All of the available 3' sequence of the bovine (B), human (H) and rat (R) GH genes and the human placental lactogen gene (HPL) were aligned with the porcine (P) GH gene sequence (2.5.3). Homology between adjacent sequences is indicated by an asterisk. The site at which the cleavage and polyadenylation of the mRNA of each gene occurs is indicated with an arrow, and the AATAAA and GT-rich sequences involved in the polyadenylation of mRNAs are indicated above the aligned sequences.

```
      5'                                                                                                   AATAAA
P     TAG TTGCTGGGCATCTCT----GTTG-----CCCC-T--CCCCAGTACCTCCCCTGACCCTGGAAAGTGCCACCCCAATGCCTGCTGTCCTT-TCCTAATAAAA
      *** ****  * ***** *     ***     **** *  **** ** *** ** *********** ******* ** *      ******** ***********
B     TAG TTGCCAGCCATCTGTT--GTTTG-----CCCC-T--CCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCA------CTGTCCTT-TCCTAATAAAA
      ***  **** *       *     *     **** *  **** ******   * ** ** ****** *********      *   **** ***********
H     TAG CTGCCCGGGTGGCATCCCTGTGA-----CCCC-T--CCCCAGTGCCTCTCCTGGCCTTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAA
      ***    * *   ***  ** *** *     **** *  ****  **        *   ** * **  *****        ***    * **************
R     TAG GCACACAC-TGGTGTCTCTGCGGCACTCCCCCGTTACCCCCCTG-------TACTCTGGCAA-CTGCCA---------CCCCTACACTTTGTCCTAATAAAA
      *** *  * *   * *  **  ** *     **** *  ****  **        *   *   **  *****        ***    * **************
HPL   TAG GTGCCCGAGTAGCATCC-TGTGA-----CCCC-T--CCCCAGTGCCTCTCTTGGCCCT-GAAGGTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAA

                          ↓         GTGTCACT
P     -CCAGG---TTGCATCGT    A-TTGTCTGAGTAGGTGTCACTCTGCGAT---GGAGGGA--GGTGGGGCAGTAGGGCAAGGGGTGGGGGTGG---GAAGACAA
        ***   ********      * ****************** ***    *   ** ***   ********** * ********  **  ***    ********
B     -TGAGGAAATTGCATCGC    A-TTGTCTGAGTAGGTGTCATTCTATTCT---GGGGGGTGGGGTGGGCAGGACAGCAAGGGGGAGGATTGG---GAAGACAA
       *    ** *******        ******** ********  *****  *        ******* * ****  *     *
H     -TT---AAGTTGCATCA     TTTTGTCTGACTAGGTGTCC-TCTATAATATTATGGGGTGGAGGGGGGTGGTTTGGAGCA/
       **   *** *******       *   **    *  * *   * * ***       ** *            * ***
R     -TT---AAGATGCATCATAT CACTCTGCTAGACATCTTTTTTTTTTTAAGGCGTCCGGTTTTTTTTTTTAGATTTATTTATTTATTATAAG/
       **   *** ** ****          * **   **  * *  ** * ***       ** *       *   * * *                **   ******
HPL   ATT---AAGTTGTATCA     TTCAT-CTGACCAGGTGTCATTCTATAATATTATGGGGTGGAAGGTGGTGGTATGGAGCAAGGGGTAGGTGGAAAGAAGACTT

                                                                                                          3'
P     CCTGCAGGCCATCCTTGGGGGTCTCCTGGGGACCTAGACA-----------CTGAATGAT------GGTTGACCC-GGCTTCTTCCTGGGCTTGAAAGAG ...
          *** *** ** **** * *  ****   ***               ****** **       ****   ** **
B     TAGC-AGG-CATGCT-GGGGATGCGGTGGGCT-CTATGGGTACCCAGGTGCTGAAGAATTGACCCGGTTCCTCCTGGG/
          *** * * *   **  *    ****   *** *            ***** * * * * ****          *****    ****  **** **
HPL   GG---AGGGCCTTCAA-GGTCTAT--TGGGAA-CTAGGCC---------ACTGAAATA-TAAGCAGGTTT---GGCTGTTCT----GGGCC-GAAA-AG/
```

CHAPTER 3

BACTERIAL EXPRESSION OF PGH

3.1 Introduction

The effectiveness of growth hormone therapy in improving the growth rates of farm animals was discussed in Chapter 1 (1.3.2). Recombinant derived bovine and human growth hormones have been shown to have equal biological potencies to pituitary derived hormone (Olson *et al.*, 1981; Kaplan *et al.*, 1986; Bauman *et al.*, 1985). The aim of the work described in this Chapter was to construct plasmids capable of expressing high levels of PGH in *E.coli*, for use as an anabolic agent in livestock production.

There are a number of important differences between the organization and expression of prokaryotic and eukaryotic genes. One important difference is the disruption of coding sequences by intervening sequences, or introns, only in eukaryotic genes (Breathnach and Chambon, 1981). The presence of these sequences in most eukaryotic genes necessitates the use of cDNA copies of eukaryotic genes for expression in prokaryotic systems. A second fundamental difference is the presence of a ribosome binding site (RBS), also known as the Shine/Dalgarno sequence, just upstream of the ATG initiation codon in prokaryotic mRNAs (Shine and Dalgarno, 1975), which is not a feature of eukaryotic mRNA. To obtain the expression of a eukaryotic gene in a prokaryotic host, a cDNA copy of the gene must therefore be provided with an efficient RBS, which is located at a suitable distance from the initiator ATG. In addition, as eukaryotic promoters do not in general function in prokaryotes a prokaryotic promoter sequence must also be provided.

The first eukaryotic proteins expressed in bacterial cells were fusion proteins. These were produced from either synthetic or cDNA copies of eukaryotic sequences which were joined to the coding region of *E.coli* proteins such as beta-galactosidase or beta-lactamase. These prokaryotic sequences provided both the promoter and RBS sequences required for bact-

erial protein production which were not present in the eukaryotic genes (e.g. Itakura *et al.*, 1977; Villa-Komaroff *et al.*, 1978; Mercereau-Puijalon *et al.*, 1978; Chang *et al.*, 1978; Fraser and Bruce, 1978; Seeburg *et al.*, 1978). Typically these fusion proteins were produced at levels of less than 1 % of the total cellular protein and after cleavage from the *E.coli* portion of the protein, could only be detected by biological or radio-immune assays. The first example of the efficient production of an unfused eukaryotic protein in *E.coli* was human GH (Goeddel *et al.*, 1979). This was achieved using a combination of synthetic and cDNA sequences to construct a hybrid HGH coding sequence which was cloned into a plasmid adjacent to an *E.coli* promoter sequence which also provided an RBS.

The parameters governing the bacterial expression of eukaryotic proteins have been studied in detail since the first reports of the production of fusion proteins which appeared almost ten years ago. Expression levels of up to 60 % of the total cellular protein can be achieved using the extremely efficient hybrid *E.coli* promoters now available such as the *tac* (de Boer *et al.*, 1983a), *trc* (Brosius *et al.*, 1985) and *rac* (Boros *et al.*, 1986) promoters. Each of these promoters contain consensus sequences from efficiently expressed *E.coli* genes joined to the *lac* operator site, which allows the expression from these promoters to be regulated in *lac* I^q *E.coli* strains (de Boer *et al.*, 1983a).

Obtaining efficient high level expression is much more difficult than originally thought, and the conditions required for high level production of any particular protein are often specific to that protein. There have been many reports which describe the lack of detectable protein production in *E.coli* despite the efficient transcription of the gene in question from powerful *E.coli* promoters (e.g. Schoner *et al.*, 1984; Buell *et al.*, 1985). The low level of protein has been found to be due to the inefficient

translation of the foreign mRNA, as alterations which yield increased levels of protein production do not alter mRNA levels (Schoner *et al.*, 1984). This lack of expression occurs despite the presence of efficient RBS sequences. The most common way production increases are engineered is by synthesizing (or mutating) the 5' coding region of the gene, incorporating codons that either a) mimic those translated with high efficiency by *E.coli* (e.g. Seeburg *et al.*, 1983; Schoner *et al.*, 1984; George *et al.*, 1985) or b) inhibit the formation of secondary structures in the mRNA (e.g. Wood *et al.*, 1984; Tessier *et al.*, 1984; Buell *et al.*, 1985).

This Chapter describes the cloning of the porcine GH cDNA insert contained in pPG.3 into a bacterial expression vector, and the manipulations which were necessary in order to obtain high level expression of PGH in *E.coli*, for use in the animal industry.

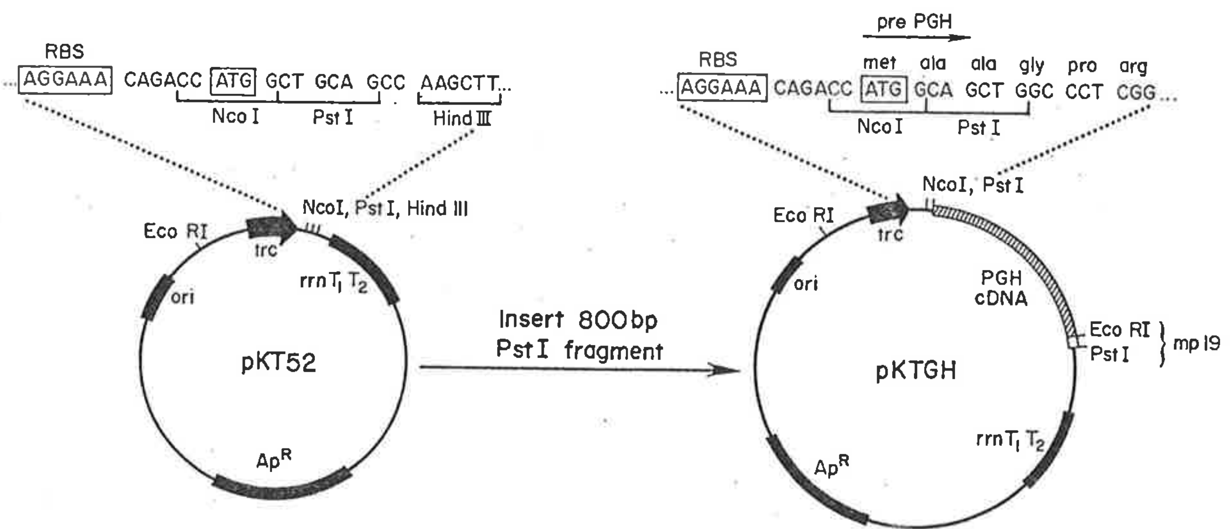
3.2 Expression of PGH in *E.coli*

The expression vector chosen for the production of PGH in *E.coli* cells was pKT52. This plasmid, kindly provided by J.Shine (California Biotechnology), is a small, high copy number expression vector which contains a powerful, regulatable *trc* promoter (Brosius *et al.*, 1985) and the strong *E.coli* 5S transcription terminators (Brosius *et al.*, 1981). The *trc* promoter is a fusion promoter containing the consensus -35 region of the *E.coli* *trp* promoter joined to the consensus -10 region of the *E.coli* *lac*UV5 promoter (de Boer *et al.*, 1983a). The *lac*UV5 sequences of this promoter contain a *lac* operator site, which results in transcription from this promoter being repressed in *lac* I^q strains. Transcription from this promoter in *lac* I^q strains can be stimulated by growing cells in the presence of IPTG (de Boer *et al.*, 1983a). A restriction map and the sequence of the RBS/ cloning region of pKT52 is shown in Figure 3-1.

FIGURE 3-1

Cloning PGH cDNA into expression vector pKT52

The organization of the *E.coli* expression vector pKT52 is illustrated. An 800 bp *PstI* fragment was isolated from M13 RF containing the *EcoRI* insert of pPG.3. This fragment contained the entire pre-PGH coding region, minus the first two amino acids, plus 33 bp of mp19 polylinker DNA. When this fragment was inserted into the *PstI* site of pKT52, the full pre-PGH sequence is regenerated (3.2.1). The *EcoRI* insert of pKT52 was then isolated and cloned into M13 mp19 to facilitate mutagenesis of the pKT52/PGH cDNA junction point (3.2.2). The position of the *trc* promoter and the 5S rRNA transcription terminators (rrnT1 and rrnT2) are indicated.


RBS
...AGGAAA CAGACC ATG GCT GCA GCC AAGCTT...
Nco I
Pst I
Hind III
NcoI, Pst I, Hind III
Eco RI
trc
ori
rrnT1 T2
pKT52
ApR
Insert 800bp
PstI fragment
pre PGH
RBS
met ala ala gly pro arg
...AGGAAA CAGACC ATG GCA GCT GGC CCT CGG...
NcoI
Pst I
NcoI, Pst I
Eco RI
trc
ori
PGH
cDNA
Eco RI
Pst I
mp19
pKTGH
rrnT1 T2
ApR

Two *E.coli* strains were used for analysing expression levels in the work descibed in this Chapter, strains MC1061 and JM101 (6.2.4). Expression from the *trc* promoter is constitutive in MC1061 cells, and repressed in the *lac* I^q strain, JM101. The repression of transcription from the *trc* promoter in JM101 cells was released by growing cells in the presence of 1 mM IPTG.

3.2.1 Pre-PGH expression plasmid, pKTGH

The initial cloning strategy was to insert the cDNA sequences encoding pre-PGH into pKT52. It has been shown previously by others that the signal sequence of human GH functions in *Pseudomonas aeruginosa*, such that it directs the secretion of mature (processed) HGH into the periplasmic space (Gray *et al*., 1984). It was therefore reasoned that the PGH signal sequence may function in *E.coli* cells and direct the secretion and processing of PGH. Recently, the HGH signal sequence has also been shown to direct the secretion and correct processing of pre-HGH in *E.coli* cells (Gray *et al*., 1985).

The nucleotide sequence of pKT52 between the initiator ATG and the *PstI* cloning site is identical to the ten bases of the PGH cDNA downstream from the initiator ATG (Figure 3-1). The cloning of a *PstI* fragment containing the entire PGH cDNA, minus the sequence upstream of the *PstI* site, into the *PstI* site of pKT52 therefore recreated the entire pre-PGH coding sequence, including the ATG, at the correct distance from the vector RBS sequence. The nucleotide sequence of the junction between the bacterial and PGH sequences in this plasmid, pKTGH, is illustrated in Figure 3-1.

Following the transformation of pKTGH into JM101 these cells were expected to produce an additional protein with a molecular weight of approximately 24,000 (pre-PGH) or, if the signal sequence was functional, 22,000 (mature, processed PGH) when induced with IPTG. As Figure 3-2 illus-

trates, this was not the case. When protein extracts prepared from cells containing pKTGH were subjected to SDS polyacrylamide gel electrophoresis (SDS/PAGE, 6.3.10) and stained with coomassie blue an identical protein banding pattern to JM101 cells containing pKT52 was produced.

To determine if the lack of pre-PGH production was due to the inefficient transcription of the PGH cDNA, RNA was isolated (6.3.9.i) from induced JM101 cells containing both pKT52 and pKTGH and the level of PGH mRNA determined by primer extension analysis using the 25 base long PGH-specific oligonucleotide, GH.25 (6.2.5; 6.3.9.iv). This analysis indicated that high levels of PGH mRNA were being produced from pKTGH (data presented below; 3.2.3).

As little was known at this time about the production, behaviour and stability of eukaryotic pre-hormones in *E.coli*, it was decided to pursue the expression of mature PGH, rather than attempting to determine what factors were responsible for the lack of expression of pre-PGH.

3.2.2 Methionyl-PGH

i) plasmid pGHX.1

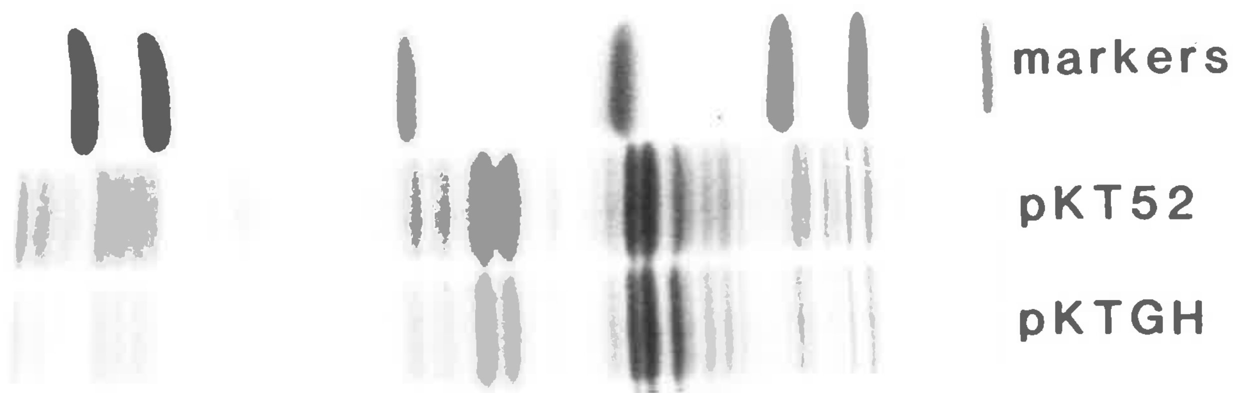
The amino-terminal residue of the mature form of PGH is a phenylalanine (Figure 2-4). To obtain expression of the mature form of PGH it was therefore necessary to delete the signal sequence and to provide the amino-terminal methionine residue essential for the initiation of translation. A construct designed to express methionyl-PGH (m-PGH) was constructed using oligonucleotide directed mutagenesis (6.3.8) to delete the DNA coding for amino acids 2-26 of the pre-hormone. This deletion joined the TTC codon of the first amino acid of the mature PGH molecule directly to the ATG initiator codon.

The *EcoRI* insert of pKTGH, which contains the entire PGH cDNA fused to the *trc* promoter sequences (Figure 3-1), was isolated and cloned into M13

FIGURE 3-2

Expression of PGH from plasmid pKTGH

JM101 cells containing either pKT52 or pKTGH were induced with 1 mM IPTG for two hours, then collected and total protein extracts prepared (3.2.1). Protein extracts were elecrophoresed on a 12.5 % SDS/polyacrylamide gel and stained with coomassie blue (6.3.10).


markers
pKT52
pKTGH

mp19 (6.3.6). Single-stranded DNA isolated from this 'phage was then used in mutagenesis reactions. To remove the 75 bases coding for amino acids 2-26 of the pre-hormone a 30 base long oligonucleotide, GH.30, complementary to 15 bases either side of the required deletion was used in a mutagenesis reaction. Following annealing and extension (6.3.8) the mutagenesis reaction was transformed into JM101. The resulting plaques were then screened to detect the required deletion by plaque hybridization of duplicate lifts made from the transformation plates (6.3.8.ii). Of the 150 plaques screened 35 % were found to hybridize in duplicate to the GH.30 oligonucleotide probe (Figure 3-3). Single-stranded DNA was isolated from a number of positive plaques and sequenced (6.3.6) to confirm the accuracy of the deletion using the PGH-specific oligonucleotide, GH.25 (6.2.5), as primer. All of the positive plaques contained DNA which had the correct deletion. The nucleotide sequence of one of these is illustrated in Figure 3-4. Replicative form (RF) DNA was isolated from this deleted 'phage (mpGHX.1) and the *EcoRI* insert purified and subcloned into the larger *EcoRI* fragment of pKTGH to create plasmid pGHX.1. The nucleotide sequence of the 5' end of this, and the other expression plasmids described in this Chapter are illustrated in Figure 3-5.

When proteins prepared from either MC1061 or induced JM101 cells containing pGHX.1 were examined by SDS/PAGE (6.3.10), no additional protein of the expected size, 22,000 daltons (22 K), was present (data discussed below). As will be discussed in detail below (3.2.3), primer extension analysis of RNA from these cells indicated that pGHX.1 was transcribing high levels of PGH mRNA.

ii) pGHX2.1 (two cistron) and pGHXF (fusion) plasmids

Experiments aimed at achieving high levels of m-bovine GH expression in *E.coli* performed by Schoner *et al.* (1984) had initial results similar to

FIGURE 3-3

Screening M13 plaques for oligonucleotide generated mutants

Duplicate nitrocellulose lifts were made off a plate containing 150 plaques from the GH.30 mutagenesis reaction (3.2.2.i; 6.3.8.ii). The lifts were hybridized to end-labelled oligonucleotide GH.30 and mutants selected by washing the discs in 3 M TMACl at 65 ^{o}C (6.3.8.iii). Autoradiography of the washed discs indicated that approximately 35 % of the plaques hybridized to the GH.30 probe in duplicate (3.2.2.i).

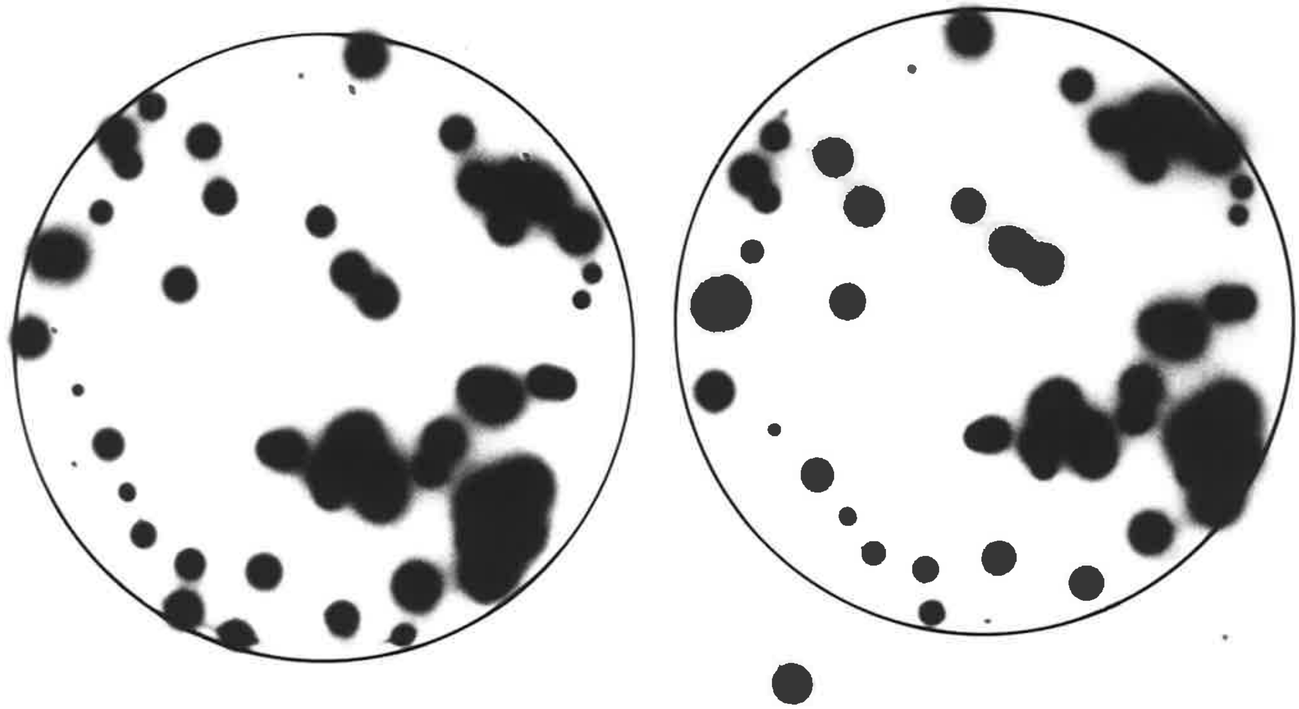
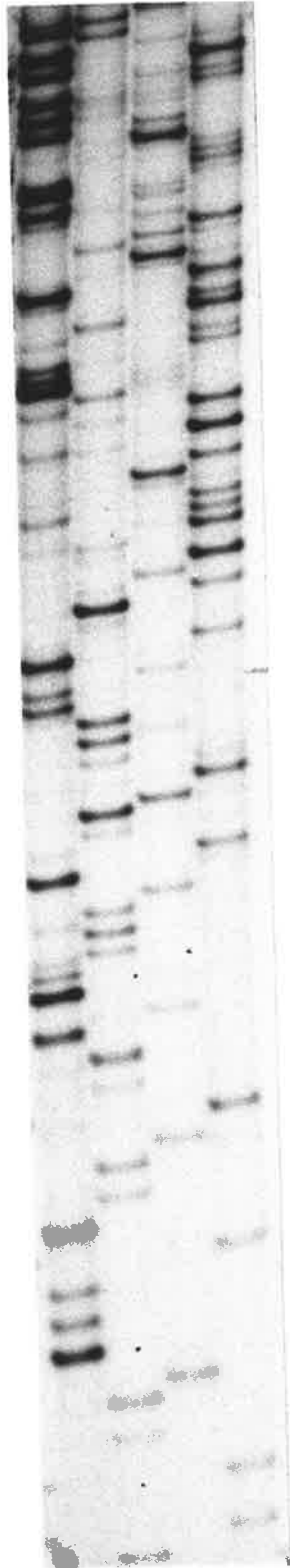


FIGURE 3-4

Nucleotide sequence of oligonucleotide GH.30 generated mutant.

The nucleotide sequence of the junction point between PGH cDNA and pKT52 sequences. This sequence was generated from single-stranded DNA purified from one of the hybridization positives shown in Figure 3-3. The sequencing reaction was primed using the PGH specific oligonucleotide, GH.25 (6.2.5), which is homologous to bases 175 to 199 of the PGH cDNA sequence (Figure 2-4). The oligonucleotide directed deletion has fused the codon encoding the first amino acid of the mature PGH molecule directly to the pKT52 initiator methionine.


T C G A

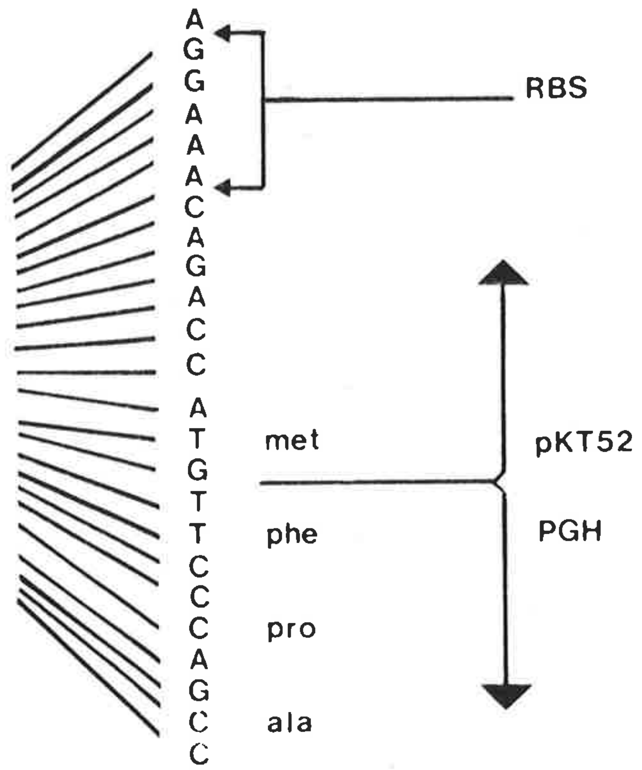

A
G
G
A
A
A
C
A
G
A
C
C
A
T
G
T
T
C
C
C
A
G
C
C
RBS
met
phe
pro
ala
pKT52
PGH

FIGURE 3-5

Nucleotide sequence of 5' regions of PGH expression plasmids

The nucleotide sequence of the final 24 bases of the mRNA leader and the 5' end of the coding region of each of the expression plasmids generated in Chapter 3 are illustrated. Each of the sequences were determined by priming sequencing reactions with the PGH specific oligonucleotide, GH.25 (Figure 3-4; 6.2.5). Each of the plasmids with the exception of pKTGH (pre-PGH) and pGHXF (PGH fusion protein) encode methionyl-PGH (m-PGH). Bases of the m-PGH expression plasmids which differ from the pGHX.1 sequence have been underlined.

```
                      RBS
                 +24
pKTGH       ...CACAGGAAACAGACC ATG GCT GCA GGC CCT CGG ACC TCC GTG CTC CTG...
                               met ala ala gly pro arg thr ser val leu leu

pGHX.1      ...CACAGGAAACAGACC ATG TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT...
                               met phe pro ala met pro leu ser ser leu phe

pGHX2.1     ...CACAGGAAACAGACC ATG GAG GAT GAT TAA ATG TTC CCA GCC ATG CCC...
                               met glu asp asp *** met phe pro ala met pro

pGHXF       ...CACAGGAAACAGACC ATG GAG GAT GAT AAC GGT TTC CCA GCC ATG CCC...
                               met glu asp asp asn gly phe pro ala met pro

pGHXS.4     ...CACAGGAGGTAATAT ATG TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT...
                               met phe pro ala met pro leu ser ser leu phe

pGHXS.9     ...CACAGGAGGTAAAAT ATG TTC CCA GCC ATG CCC TTG TCC AGC CTA TTT...
                               met phe pro ala met pro leu ser ser leu phe

pGHXC.1     ...CACAGGAAACAGACC ATG TTC CCA GCC ATG CCG CTG TCC AGC CTG TTC...
                               met phe pro ala met pro leu ser ser leu phe

pGHXSC.4    ...CACAGGAGGTAATAT ATG TTC CCA GCC ATG CCG CTG TCC AGC CTG TTC...
                               met phe pro ala met pro leu ser ser leu phe
```

those described above i.e. high levels of transcription but low (1.7 % of total) protein production. These authors found that production levels could be elevated (from 1.7 % to 34 % of cell protein) by expressing BGH as a fusion protein containing a number of additional amino-terminal amino acid residues. These authors also found that production levels still remained high (19 % of total cellular protein) if a stop codon was inserted between the additional amino-terminal residues and the BGH coding sequences. This expression of the BGH apparently occurs due to read-through by the E.coli ribosomes which fail to stop at the first termination codon. The first cistron also contains a consensus RBS which may allow independent initiation at the BGH cistron. Schoner et al. (1984) have described this as a two-cistron expression system.

To apply these results to the expression of m-PGH, an oligonucleotide was designed to insert a short second cistron directly in front of the m-PGH coding sequence present in pGHX.1. The second cistron encoded the sequence met-glu-asp-asp-stop, and contained within this sequence nucleotides homologous to the RBS consensus (see above). This was based on the sequences Schoner et al. (1984) had demonstrated to enhance the translation of BGH. The nucleotide sequence and the resulting translation product of this insert is shown in Figure 3-5. The 15 bases encoding the second cistron codons were inserted directly in front of the ATG initiation codon of pGHX.1 using a 50 base oligonucleotide, GH.50 (6.2.5). This oligonucleotide contained sequences homologous to 15 bases either side of the required insertion, plus sequence complementary to the second cistron. The EcoRI insert of pGHX.1 which had been cloned into M13 was mutagenized as described (6.3.8) and the resulting plaques screened for insertions by plaque hybridization. Approximately 35 % of the screened plaques were found to be positive, and a number of these were purified and sequenced (6.3.7). The

EcoRI insert isolated from RF DNA of 'phage containing the correct insertion was subcloned (6.3.4.iii) into the EcoRI fragment of pKTGH to create pGHX2.1. Transformation of this plasmid into both MC1061 and induced JM101 cells failed to result in the expression of m-PGH levels high enough to be detected by SDS/PAGE analysis (6.3.10) of protein extracts.

To examine the possibility that the stop/start nature of the two cistron expression system was responsible for the lack of m-PGH expression, a further mutagenesis step was performed which replaced the TAA/ATG stop/start codons (Figure 3-5) with a sequence encoding asn-gly. This created a plasmid which should express a fusion protein, the five amino acid non-PGH portion of which could be removed by chemical cleavage with hydroxylamine (Bornstein and Balian, 1977). The two codon substitution was performed on an M13 clone containing the EcoRI insert of pGHX2.1 by the procedure described above, using the 42 base long oligonucleotide GH.42 (6.2.5), resulting in 6 % of the plaques containing the correct alteration (see Figure 3-5 for sequence). After purifying and subcloning sequences with the correct substitution into pKTGH to create plasmid pGHXF, examination of protein lysates from transformed E.coli cells was performed (6.3.10), but failed to demonstrate the presence of a PGH fusion protein.

iii) pGHXS (spacer) plasmids

The production of a number of proteins in E.coli has been found to be inefficient due to the formation of stable secondary structure in the mRNA around the translation initiation region (e.g. Wood et al., 1984; Tessier et al., 1984; Buell et al., 1985). Computer analysis of the PGH mRNA sequence was performed using the Staden programs HAIRPN and HAIRGU, but no convincing structures around this region were evident. A number of other reports have also indicated that coding sequences between the RBS and the initiator ATG, hereafter referred to as the "spacer", can also influence

translational efficiency (Gheysen et al., 1982; de Boer et al., 1983b; Hui et al., 1984; George et al., 1985; Whitehorn et al., 1985). As the sequence of the spacer of pKT52 differed greatly from those found to be efficient in other systems it was decided to alter the spacer region of expression plasmid pGHX.1 to create a consensus RBS and an AT-rich spacer region, based on sequences defined in the above references and the RBS consensus (Scherer et al., 1980).

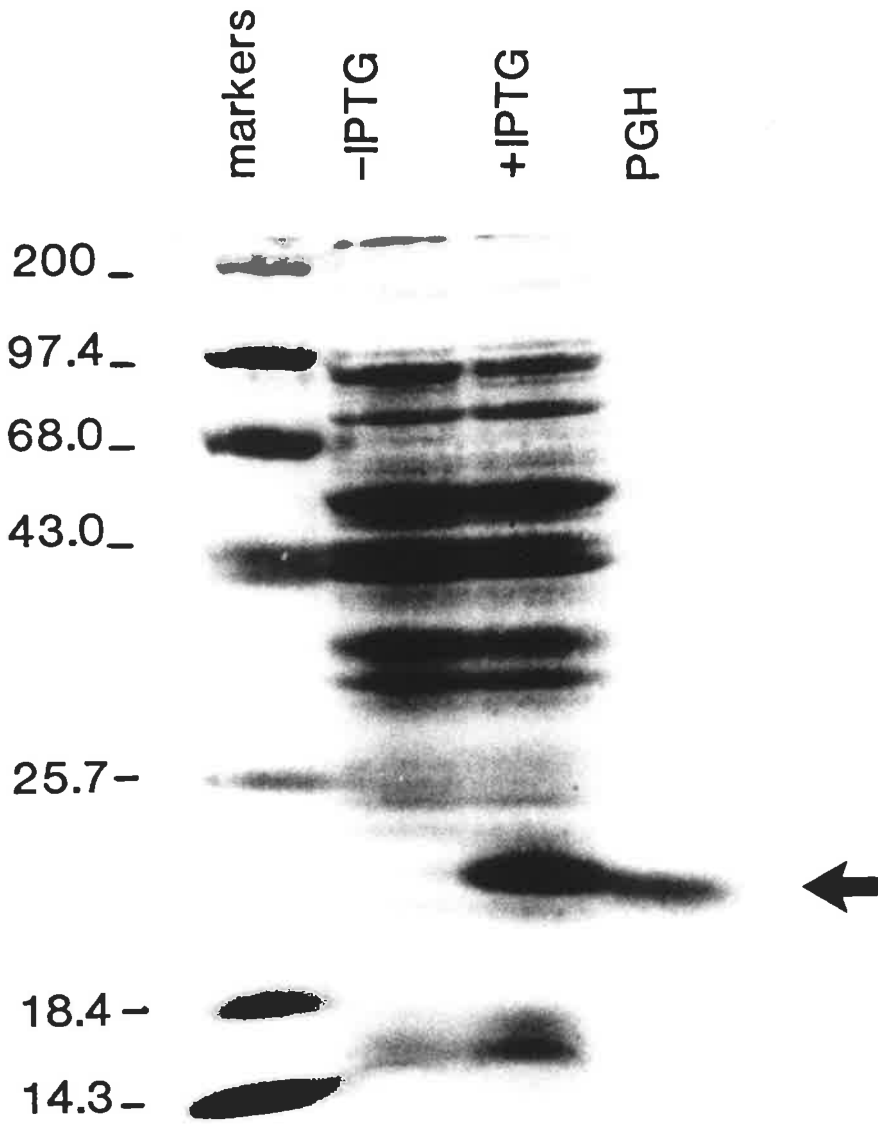
A 38 base oligonucleotide with one redundancy, GH.38 (6.2.5), was designed to alter the RBS from AGGAAA to AGGAGG and the spacer from CAGACC to either TAATAT or TAAAAT. Single-stranded DNA containing the small EcoRI fragment of pGHX.1 was mutagenized as described (6.3.8) and positive plaques selected and sequenced. A total of 30 % of the plaques hybridized to the GH.38 probe, but of these only 20 % contained either the GGTAATAT or GGTAAAAT RBS/spacer sequences. The remaining positives contained duplicate insertions which may have arisen due to the incorrect hybridization of the GH.38 oligonucleotide during the mutagenesis reaction. RF DNA was prepared from plaques containing correct versions of both the required alterations and then cloned back into pKTGH. The nucleotide sequence of the RBS/spacer region of the resulting plasmids, pGHXS.4 and pGHXS.9 are illustrated in Figure 3-5. Extracts prepared from both MC1061 and induced JM101 cells containing either of these two plasmids were found to contain an additional prominent band, which had a molecular weight of 22 K, the expected size of m-PGH. Laser densitometry of SDS/PAGE gels indicated that both plasmids produced identical levels of m-PGH, which ranged in the two hosts from 15 to 20 % of total cellular protein.

Figure 3-6 illustrates the level of m-PGH produced from pGHXS.4 in both uninduced and induced JM101 cells. This Figure also illustrates the similarity in molecular weight of the E.coli produced protein versus pitu-

FIGURE 3-6

Production of m-PGH by plasmid pGHXS.4

Protein extracts from uninduced (-IPTG) and induced (+IPTG) JM101 cells containing expression plasmid pGHXS.4 were subjected to SDS/PAGE, along with molecular weight markers and purified, pituitary derived PGH (kindly provided by R. Seamark). A prominent band of the expected molecular weight of PGH (22,000 daltons) is produced only in IPTG induced cells (3.2.2.iii).


markers
-IPTG
+IPTG
PGH
200
97.4
68.0
43.0
25.7
18.4
14.3

itary derived PGH. The m-PGH migrates at a slighty higher molecular weight than expected (approximately 200 daltons), probably due to the extremely crude nature of the protein extracts applied to the gel. This phenomenon has been previously observed in crude bacterial extracts containing human GH (Hsiung *et al.*, 1986).

iv) pGHXC.1 (codon) and pGHXSC.4 (spacer plus codon) plasmids

The inefficient translation of a number of proteins in *E.coli* has been overcome in a number of instances by the alteration of codons in the 5' end of the foreign gene, so that they resemble those used in efficiently expressed *E.coli* proteins. An example of this is the expression of m-PGH in *E.coli* described by Seeburg *et al.* (1983). These authors replaced the entire 5' end of the PGH gene with a synthetic DNA sequence incorporating codons known to be efficiently translated.

Analysis of the 5' end of the PGH cDNA sequence indicated that there were a number of codons present which were infrequently used in *E.coli* genes (Maruyama *et al.*, 1986). In particular the two leu codons encoding residues 7 and 10 of m-PGH are very rarely used in *E.coli* genes. These two codons plus the adjoining pro and phe codons at residues 6 and 11, were replaced with codons used frequently in highly expressed *E.coli* genes using a 34 base oligonucleotide, GH.34 (6.2.5). This oligonucleotide was homologous to the 5' end of the PGH gene but contained four scattered base mismatches which would alter these four codons to the required sequences.

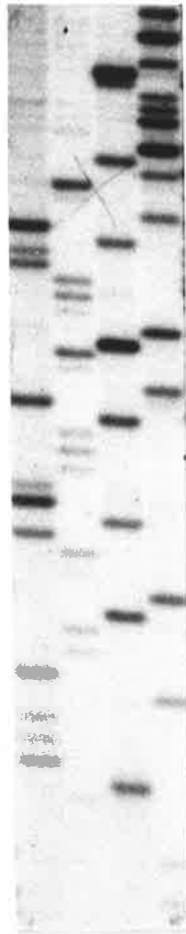
M13 clones containing the coding sequences of both pGHX.1 and the spacer altered plasmid, pGHXS.4 were mutagenized with oligonucleotide GH.34 (6.3.8), mutants selected by plaque hybridization, and their altered structure confirmed by nucleotide sequencing. A sequencing gel demonstrating the presence of the altered codons in one of the pGHX.1 positive plaques as compared to the parental sequence is shown in Figure 3-7. The nucleotide

FIGURE 3-7

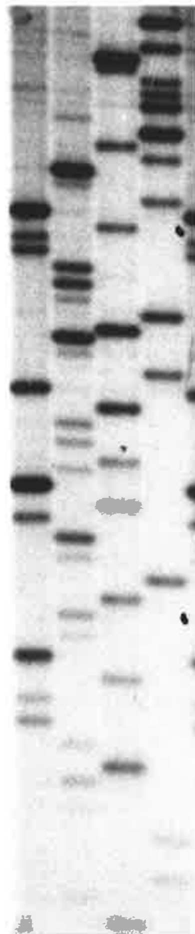
Nucleotide sequence of codon alterations in PGH expression plasmids

The nucleotide sequences of the inserts of plasmids pGHX.1 (PGH codons, A) and pGHXC.1 (containing four altered, *E.coli*-like, codons, B) are shown (3.2.2.iv). The sequences were generated using the PGH specific oligonucleotide, GH.25 (6.2.5). The four single base changes which result in the alteration of four codons in pGHXC.1 are indicated.

A



B



sequences of the 'phage containing these substitutions are illustrated in Figure 3-5. RF DNA was prepared from the appropriate plaques and the EcoRI inserts isolated and cloned back into pKTGH. The pGHX.1 codon altered sequence was named pGHXC.1 and the pGHXS.4 codon altered sequence named pGHXSC.4. SDS/PAGE analysis of protein extracts prepared from induced JM101 cells (6.3.10) containing these plasmids indicated that only pGHXSC.4 contained detectable levels of m-PGH, and that the level of m-PGH production was no greater than pGHXS.4. The level of m-PGH produced by these two plasmids and other PGH expression plasmids are illustrated in Figure 3-8.

3.2.3 Analysis of transcription levels

To determine if the increased levels of protein production by plasmids pGHXS.4 and pGHXSC.4 were due to the enhancement of transcription or translation of the PGH mRNA, RNA was isolated (6.3.9.i) from MC1061 cells containing the expression plasmids pKT52, pKTGH (pre-PGH), pGHX.1 (m-PGH), pGHXC.1 (codon altered), pGHXS.4 (RBS/spacer altered) and pGHXSC.4 (RBS/spacer and codon altered). The PGH mRNA levels were measured by primer extension using ^{32}P-labelled oligonucleotide GH.25 as the primer. 3 ug of bacterial RNA was annealed to 4 ng of GH.25 primer, followed by enzymatic extension with reverse transcriptase. Following separation by electrophoresis through a polyacrylamide/urea gel, extension products were visualized by autoradiography (6.3.9.iv).

The control plasmid, pKT52, produced no detectable extension product, while each of the m-PGH expression plasmids produced a 115 base long extension product of similar intensity. This indicates that the increased level of m-PGH production from plasmids pGHXS.4 and pGHXSC.4 is due to an increase in translational efficiency (Figure 3-9). The band produced by pKTGH is 75 bases longer than that of the m-PGH expression plasmids (190 versus 115) due to the presence of sequences encoding the PGH signal peptide in

FIGURE 3-8

PGH protein expression levels from different expression vectors

Protein extracts from IPTG induced JM101 cells containing PGH expression plasmids (3.2.1, 3.2.2) were subjected to SDS/PAGE and stained with coomassie blue. Detectable levels of m-PGH are present only in extracts from cells containing plasmids which contain the RBS/spacer alteration, pGHXS.4 and pGHXSC.4 (3.2.2.iii).

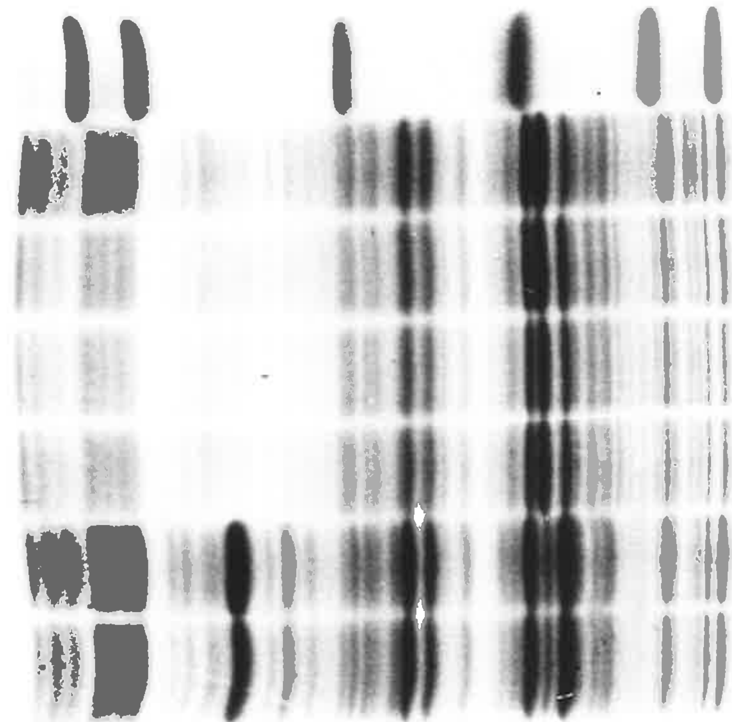

markers
pKT52
pKTGH
pGHX.1
pGHXC.1
pGHXS.4
pGHXSC.4

FIGURE 3-9

Transcription levels from different PGH expression plasmids

RNA was isolated from MC1061 cells containing the PGH expression plasmids and the PGH mRNA levels analysed by primer extension (3.2.3). Each extension reaction contained 3 ug of bacterial RNA and 4 ng of ^{32}P-kinased oligonucleotide GH.25 (6.3.9.iv; 6.2.5). Extension products were separated on a 12 % polyacrylamide/8 M urea gel and visualized by autoradiography at -80 ^{o}C for one hour.

markers
pKT 52
pKTGH
pGHX .1
pGHXC .1
pGHXS .4
pGHXSC .4

this plasmid.

3.3 Discussion

This Chapter describes the subcloning of the PGH cDNA into a bacterial expression vector and the manipulations which were required to achieve high level expression of m-PGH in *E.coli*. Initial experiments demonstrated that the PGH mRNA was transcribed efficiently from the *trc* promoter, but that no protein was produced due to the poor translation of the PGH mRNA. It was demonstrated that neither the two cistron expression system of Schoner *et al.* (1984; 3.2.2.ii) or the replacement of codons in the 5' end of the gene (3.2.2.iv) alleviated this problem. However it was shown that the optimization of the RBS/spacer region increased m-PGH production levels to 15 % of total cellular protein (3.2.2.iii).

The approach used to obtain high level expression differs from those usually employed. The most common reason for the low translation of eukaryotic genes is the formation of secondary structures in the hybrid mRNA blocking the access of ribosomes to the RBS (Steitz, 1979; Gold *et al.*, 1981; Wood *et al*, 1984; Tessier *et al*, 1984; Buell *et al*, 1985; Stanssens *et al*, 1985). Such secondary structures have been removed by targeted mutagenesis of either coding or non-coding regions in a number of cases where the sequences involved in the formation of such structures could be identified by computer analysis (see above references). However in many cases, including the experiments described in this Chapter, no obvious secondary structures are evident. In order to overcome the low translation of the PGH mRNA in *E.coli*, the spacer/RBS region of expression plasmids was altered to create a consensus RBS, and an AT rich spacer sequence which would be unlikely to be able to form any stable secondary structure. The generation of a "structureless" spacer sequence may be a useful approach for the expression of other eukaryotic genes which are translated with a

low efficiency in prokaryotic cells.

The plasmids whose construction is described in this Chapter are currently being used for small scale fermenter production of m-PGH, and are producing 1-2 gm of m-PGH per litre of culture. The material produced in this fashion has been shown to behave in an identical manner to pituitary PGH in ion-exchange chromatography (M.Snoswell pers. comm.) but the identity of the protein has not yet been confirmed by protein sequencing. When sufficient amounts of material are available, trials will begin to determine the growth promoting effects of this protein in farm animals.

CHAPTER 4

EXPRESSION OF PGH IN TRANSGENIC ANIMALS

4.1 Introduction

The genes encoding a number of different proteins from a wide range of organisms have been introduced into the germ line of transgenic mice (1.4.1; 1.4.2). Many of the promoters of the introduced genes, often fused to non-related "reporter" coding sequences, have been found to be correctly regulated in transgenic mice, such that the foreign gene is expressed predominantly in the appropriate tissues. Examples of such regulated expression have been described for genes as diverse as mouse metallothionein (Brinster et al., 1981; Palmiter et al., 1982a, 1982b, 1983), mouse immunoglobulins (Brinster et al., 1983; Storb et al., 1984; Grosschedl et al., 1984), rat elastase-I (Swift et al., 1984; Ornitz et al., 1985; MacDonald et al., 1986), rat myosin light-chain 2 (Shani, 1985), mouse (Chada et al., 1985; Magram et al., 1985) and human beta-globins (Townes et al., 1985), rat (Hanahan, 1985) and human (Bucchini et al., 1986; Selden et al., 1986) insulin, mouse alpha A-crystallin (Overbeek et al., 1985), mouse alpha-fetoprotein (Krumlauf et al., 1985), mouse major histocompatibility complex (Le Meur et al., 1985; Yamamura et al., 1985; Pinkert et al., 1985; Bieberich et al., 1986), mouse alpha 2-type I collagen (Khillan et al., 1986), the Rous sarcoma virus long terminal repeat (Overbeek et al., 1986), and the rat skeletal actin (Shani, 1986) genes. However not all genes which are introduced into transgenic mice are expressed in a predictable manner. An example of this particularly relevant to the work described in this Chapter is the human growth hormone (HGH) gene, which has been found not to be expressed in transgenic mice in two independent studies (Wagner et al., 1983; Hammer et al., 1984). In addition, the fusion of unrelated coding and promoter sequences, such as in MT-I/GH constructs (1.4.1), or the introduction of heterologous genes (e.g. human gamma-globin gene (Chada et al., 1986; Kollias et al., 1986)) may generate novel expression patterns

in some instances.

The introduction of foreign genes linked to the promoter sequences of "house keeping" genes such as the mouse metallothionein-I (MT-I) gene (Brinster et al., 1981; Palmiter et al., 1982a, 1982b, 1983; 1.4.1, 1.4.2) or the H-2K major histocompatibility gene (Morello et al., 1986; 1.4.2) has been demonstrated to be a useful approach to obtaining high level expression of the gene of interest in a range of transgenic mouse tissues. The linkage of the genes encoding rat or human GH or human GHRF genes to such promoters, followed by their introduction into transgenic mice (1.4.1), has been demonstrated to result in vastly increased growth rates (1.4.2). It is interesting to note that the introduction of a fusion gene containing the rat elastase-I promoter fused to the HGH gene failed to stimulate growth in transgenic mice, even though high levels of HGH mRNA were synthesized in the pancreas (Ornitz et al., 1985). This lack of growth was presumed to result from the absence of HGH secretion into the bloodstream.

This Chapter describes experiments which examine the possibility of utilizing gene transfer techniques for improving animal growth. As was discussed earlier (1.5), the pig was the farm animal chosen for these experiments due to its relatively high fertility and ability to bear multiple offspring. Before PGH constructs were introduced into pigs they were first used to generate transgenic mice, in order to test the effectiveness of the constructs in promoting growth in a system which could be easily analysed.

All of the animal handling and microinjection which is described in this Chapter was performed by A.Michalska and R.Ashman (Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide).

4.2 The construction of a human MT-IIA promoter/ PGH fusion gene

The promoter chosen for these studies was the human metallothionein II-A promoter (Karin and Richards, 1982) which was kindly provided by R. Richards. This is an efficient promoter from which transcription can be induced by both heavy metals and glucocorticoids (Karin *et al.*, 1984a; Karin *et al.*, 1984b). Much is known about the regulation of this promoter, and the sequences involved in the enhancement of transcription in response to inducers (Karin *et al.*, 1984b; Haslinger and Karin, 1985; Scholer *et al.*, 1986). As the hMT-IIA promoter has been shown to be active in a wide range of tissue culture cell lines (Haslinger and Karin, 1985) it was expected that it would be active in porcine tissues.

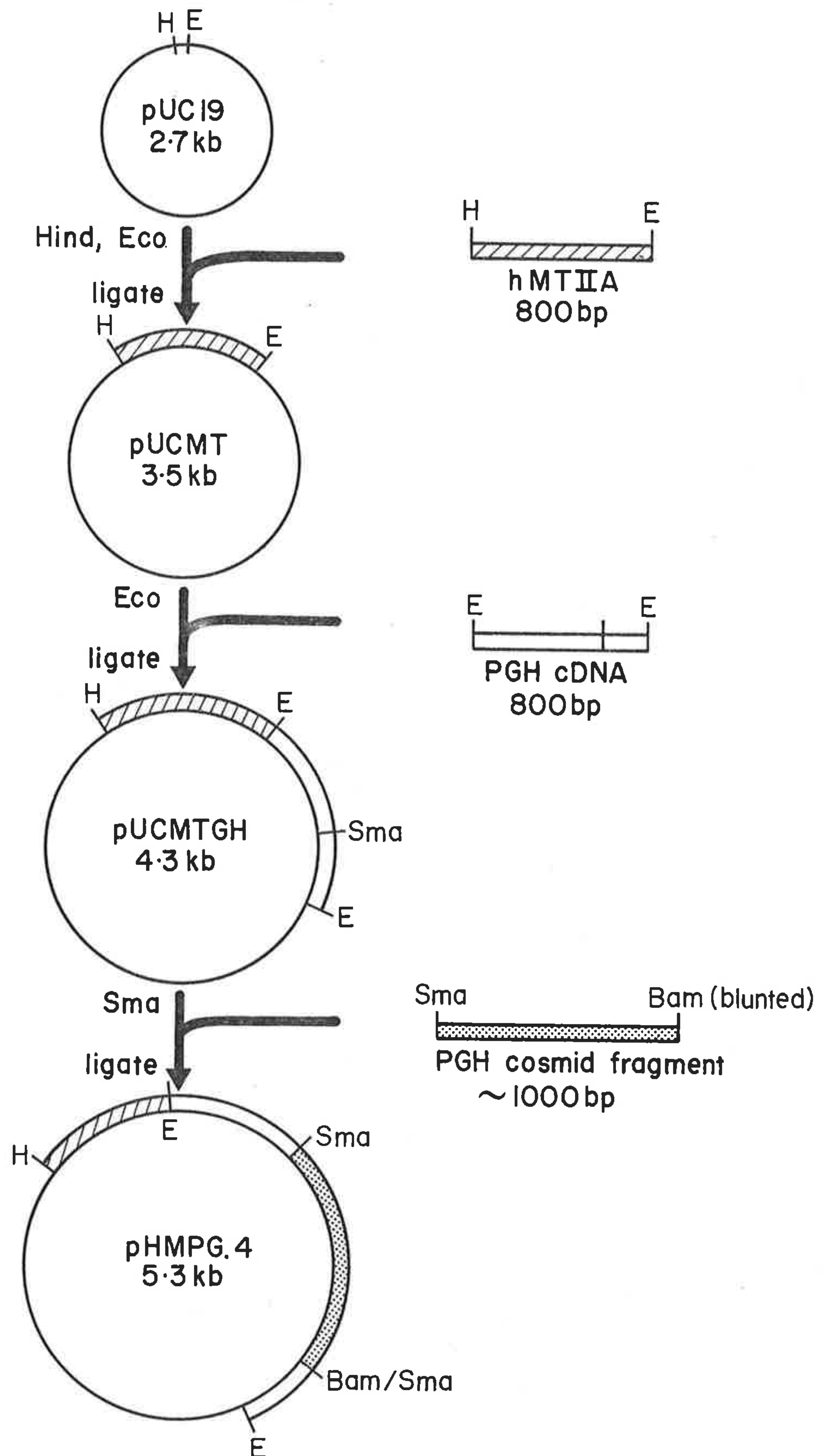
The hMT-IIA promoter was available as an 823 bp fragment with promoter sequences extending from -763 to +60 cloned in the M13 vector mp8 (A.Robins pers. comm.). The double digestion of RF 'phage containing this promoter with *Hin*dIII and *Eco*RI releases the promoter sequences fused to 5 bp of vector polylinker sequence. This 823 bp fragment was purified (6.3.2.ii) and subcloned into *Hin*dIII/*Eco*RI digested pUC19 (6.3.4.iii), to generate plasmid pUCMT.

The sequences encoding PGH were isolated from the PGH cDNA clone pPG.3 (2.2.3) as an 814 bp *Eco*RI fragment. This was cloned downstream of the hMT-IIA promoter, by restricting pUCMT with *Eco*RI, followed by ligation to the purified pPG.3 insert (6.3.4.iii). Restriction analysis of plasmid DNA prepared from the resulting transformants (6.3.4.ii) identified a plasmid which contained the PGH cDNA inserted in the correct orientation, which was named pUCMTGH.4 (Figure 4-1). The nucleotide sequence of the junction point between these two fragments was determined by the directional subcloning of a restriction fragment spanning this region into M13 mp18. The sequence data derived from this clone indicated that the expected sequence had been

FIGURE 4-1

Construction of eukaryotic expression plasmid pHMPG.4

A flow chart illustrating the construction of pHMPG.4 is illustrated (4.2). An 823 bp *Hin*dIII/*Eco*RI fragment containing the hMT-IIA promoter was cloned into *Hin*dIII/*Eco*RI digested, dephosphorylated pUC19 (6.3.4.iii) to create plasmid pUCMT. This plasmid was then restricted with *Eco*RI, dephosphorylated, and ligated to the *Eco*RI insert of the PGH cDNA clone, pPG.3 (2.2.3), to generate plasmid pUCMTGH.4. This plasmid was restricted with *Sma*I, dephosphorylated, and ligated to the blunt-ended 1 kb *Sma*I/*Bam*HI insert of cosmid subclone pGHB.3, which contains most of the last exon of the PGH genomic gene, and approximately 700 bp of PGH 3' non-coding sequence.



H E
pUC19
2·7kb
Hind, Eco
ligate
H
E
hMTIIA
800bp
H
E
pUCMT
3·5kb
Eco
ligate
E
E
PGH cDNA
800bp
H
E
pUCMTGH
4·3kb
Sma
E
Sma
ligate
Sma
Bam (blunted)
PGH cosmid fragment
~1000bp
H
E
Sma
pHMPG.4
5·3kb
Bam/Sma
E

generated, and contained the hMT-IIA promoter sequences and transcription start site (down to position +60) joined to the PGH 5' untranslated region (from +21 onwards) by 9 bp of polylinker/synthetic linker DNA (Figure 4-2).

Shortly after the isolation of pPG.3, and the generation of pUCMTGH.4, a number of publications appeared which described the involvement of sequences downstream of the polyadenylation site in the processing of poly A^+ mRNAs (Woychik et al., 1984; Gil and Proudfoot, 1984; McLauchlan et al., 1985). As these sequences are not present in cDNA clones, and the purification of the PGH genomic gene was near completion (2.4), it was decided to insert the 3' downstream sequences from the cosmid PGH gene into pUCMTGH.4 to ensure the efficient polyadenylation of the PGH fusion gene.

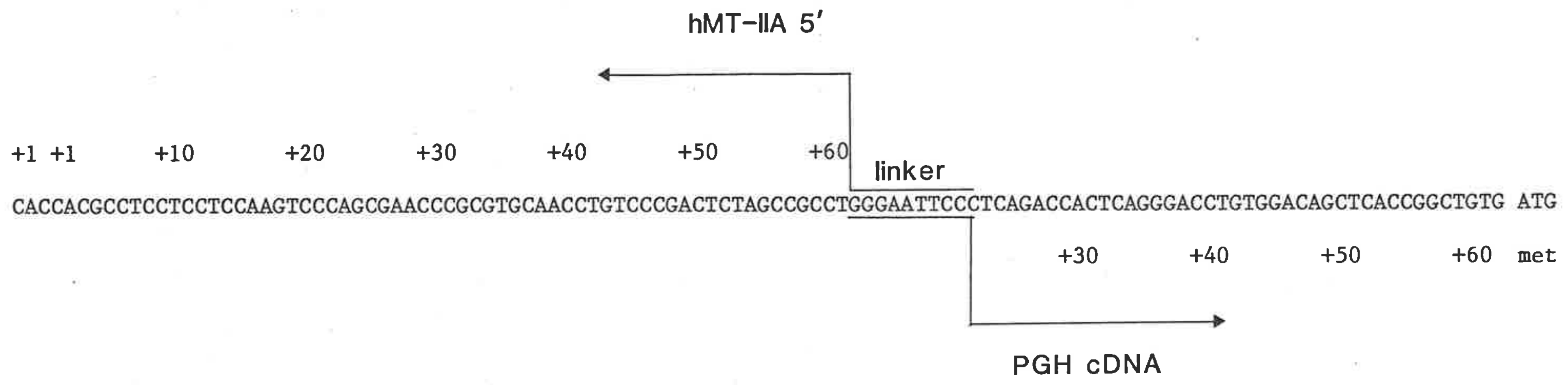
Analysis of the pPG.3 DNA sequence downstream of the stop codon failed to reveal any restriction sites which would allow the convenient insertion of a suitable restriction fragment (Figure 2-4). Preliminary sequencing data generated from a subclone of the cosmid PGH gene indicated that this gene was identical in sequence to the cDNA clone downstream of the unique SmaI restriction site, which is present in the last exon of the gene (Figure 2-9). It was therefore decided to subclone the 1 kb SmaI/BamHI fragment from plasmid pGHB.3 (2 kb BamHI subclone of cPGH.1; Figure 2-7), which contains the fifth exon downstream of the Sma I site plus approximately 800 bp of 3' non-translated sequence from the PGH gene (Figures 2-7 and 2-8) into the unique SmaI site of pUCMTGH.4.

The 1 kb SmaI/BamHI fragment of pGHB.3 was purified (6.3.2.ii), and the BamHI generated overhang repaired with the Klenow fragment of E.coli DNA polymerase I (6.3.4.iii). This blunt-ended fragment was then subcloned (6.3.4.iii) into SmaI digested pUCMTGH.4. The examination of plasmid DNA isolated from a number of the resulting transformants (6.3.4.ii) revealed that most were equal in size to pUCMTGH.4. The transformants were therefore

FIGURE 4-2

Nucleotide sequence of the hMT-IIA/PGH junction point in pUCMTGH.4

A *ScaI/PstI* fragment which covers the junction region between hMT-IIA and PGH cDNA sequences was isolated from pUCMTGH.4 and cloned into *SmaI/PstI* digested mp18 for sequence analysis. The sequence data from this clone indicated that the correct fusion had been generated, with the hMT-IIA promoter, cap site (there are two cap sites, both indicated as +1), and 5' untranslated sequences (extending down to +60), linked to the PGH cDNA sequences, which extend downstream from base +21, by 9 bp of synthetic linker sequence (4.2).


hMT-IIA 5′
+1 +1 +10 +20 +30 +40 +50 +60
linker
CACCACGCCTCCTCCTCCAAGTCCCAGCGAACCCGCGTGCAACCTGTCCCGACTCTAGCCGCCTGGGAATTCCCTCAGACCACTCAGGGACCTGTGGACAGCTCACCGGCTGTG ATG
+30 +40 +50 +60 met
PGH cDNA

screened for the presence of the cPGH.1 sequences by filter hybridization (6.3.4.i), using a 500 bp BamHI/PstI restriction fragment from the far 3' end of pGHB.3 as the hybridization probe (Figure 2-7). Restriction analysis of plasmid DNA prepared from the resulting positives (6.3.4.ii) indicated that one contained the inserted fragment in the correct orientation. This plasmid was named pHMPG.4 (HM, human metallothionein; PG, porcine growth hormone). The organization of this plasmid is illustrated in Figure 4-3.

4.3 The production of transgenic mice with pHMPG.4

4.3.1 Generating transgenic mice

The effectiveness of the pHMPG.4 construct in producing transgenic animals and promoting growth was tested by the generation of transgenic mice. As the production of transgenic farm animals was expected to be difficult, it was necessary that the construct would be both efficiently integrated, and efficiently expressed. These factors could most clearly be demonstrated using the transgenic mouse system.

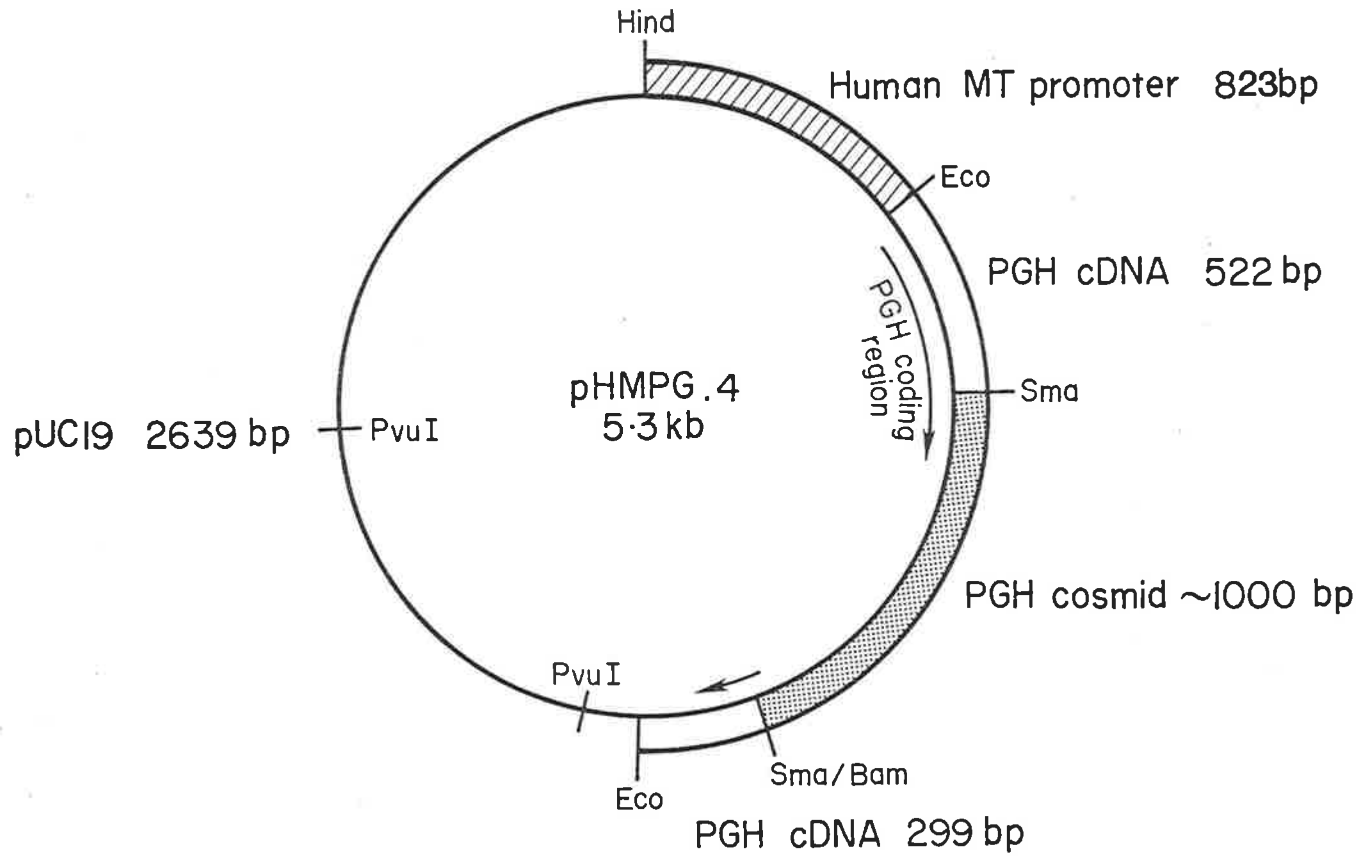
There have been a number of reports which indicate that the presence of prokaryotic vector sequences may inhibit the expression of foreign genes in transgenic mice (e.g. Brinster *et al.*, 1985; Shani, 1986). To remove vector sequences from pHMPG.4, a HindIII/PvuI digest was performed, and the 2.7 kb insert (Figure 4-3) isolated from a low melting temperature agarose gel (6.3.2.ii). Digestion with these two enzymes releases the hMT-IIA/PGH fusion gene with an additional 120 bp of the bacterial lac-Z gene attatched to the 3' end. The vector DNA, which is also 2.7 kb in length, is conveniently cleaved into two smaller fragments by these enzymes (Figure 4-3).

The linear 2.7 kb HindIII/PvuI fragment of pHMPG.4 was diluted to a concentration of 2 ng/ul, and about 1 pl of this solution (containing approximately 600 copies of the insert) microinjected into the male pro-

FIGURE 4-3

Expression vector, pHMPG.4

The restriction map and organization of expression vector pHMPG.4 is illustrated (4.2). The hMT-IIA promoter sequences are fused to a hybrid gene containing PGH cDNA sequences extending from +21 down to the unique *SmaI* site, joined to PGH genomic gene sequences downstream from this point. The 2.7 kb *HindIII*/*PvuI* fragment containing all of the promoter and PGH sequences, plus 120 bp of the pUC19 *lacZ* gene joined to the 3' end, was purified from low melting temperature agarose (6.3.2.ii), and used for generating transgenic animals.


PORCINE GROWTH HORMONE EXPRESSION VECTOR, pHMPG.4
Hind
Human MT promoter 823bp
Eco
PGH cDNA 522 bp
PGH coding region
Sma
pHMPG.4
5·3 kb
pUC19 2639 bp
PvuI
PGH cosmid ~1000 bp
PvuI
Sma/Bam
Eco
PGH cDNA 299 bp

nuclei of *in vivo* fertilized C57xCBA F2 mouse eggs (6.3.11.i). A total of 867 eggs were injected, and those which survived were transferred to the oviducts of 23 recipient CBAxC57 F1 foster mothers. Of the transferred eggs, 188 implanted, and resulted in the birth of 79 pups.

4.3.2 Analysis of integrated sequences of transgenic mice

i) Dot-blot analysis

Of the 79 mice born, 66 survived to weaning, and of these 64 were tested for the presence of the hMT-IIA/ PGH fusion gene by dot-blot analysis. Tails were removed from mice at between four to five weeks of age and the DNA purified as described (6.3.1.ii). Dot-blots (6.3.2.iv) containing samples of this DNA were hybridized to the 500 bp *Bam*HI/*Pst*I fragment of pGHB.3 which was radioactively labelled by nick-translation (6.3.5.i). This fragment corresponds to the PGH 3' non-translated sequences, extending downstream from +1951 (Chapter 2, Figure 2-9). Autoradiographs of this dot-blot revealed that 20 of the 64 mice (31 %) contained sequences homologous to the PGH hybridization probe (Figure 4-4).

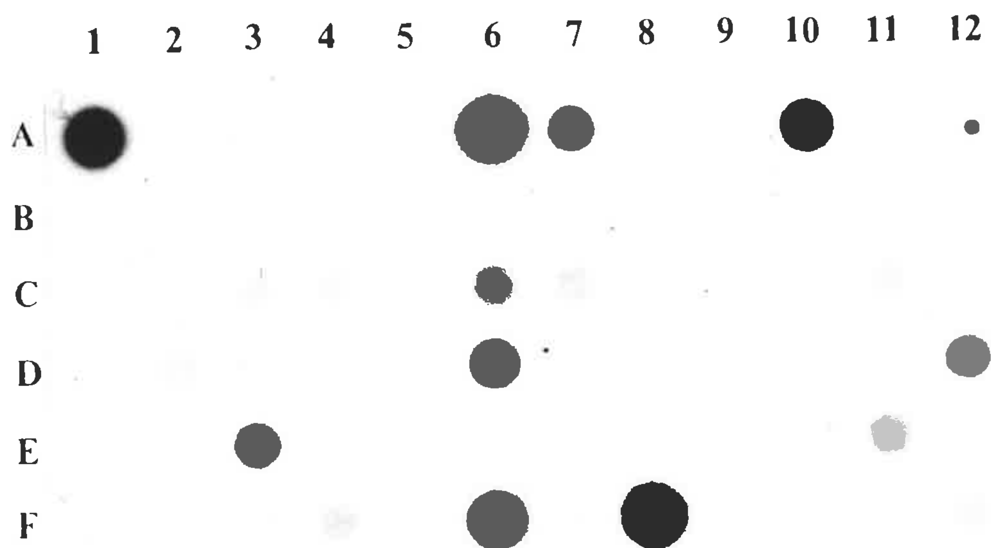
The pig DNA sample which acted as the positive control on this dot-blot hybridized very strongly to the pGHB.3 *Bam*HI/*Pst*I fragment probe (Figure 4-4). As the PGH gene appears to exist as a single copy in the porcine genome (2.5.2) this indicates that the fragment used as the hybridization probe may contain a sequence which is highly repeated, specifically in the porcine genome (the probe did not hybridize to non-transgenic mouse DNA, or in other experiments, to sheep DNA). This conclusion was later supported by Southern blots which indicated that this fragment was homologous to sequences present throughout the porcine genome (data not shown). To avoid the inconsistency of this probe with the positive control DNA, most of the remaining dot-blots utilized a *Hin*dIII/*Ava*I fragment from the 5' end of the hMT-IIA promoter, which extends from -763 to -223 (Karin and

FIGURE 4-4

Analysis of potentially transgenic mice by dot-blot hybridization

2.5 ug samples of DNA isolated from the tails of mice which developed from eggs microinjected with the pHMPG.4 HindIII/PvuI insert were denatured and applied to Gene-screen hybridization membrane (6.3.2.iv) along with pig (PIG) and mouse (MSE) positive and negative controls. A sample containing denatured pHMPG.4 plasmid DNA was also included as a second positive control. This membrane was hybridized to the nick-translated (6.3.5) 500 bp BamHI/PstI insert of pGHB.3 (Figure 2-7). This fragment contains PGH gene 3' non-translated sequences between 200 and 700 bp downstream of the poly A tail addition site. Following washing at high stringency, autoradiography of this membrane indicated that a number of the mice contained sequences homologous to the PGH gene probe (4.3.2.i). A legend indicating the identity of samples on the dot-blot is shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PIG	MSE	1	2	3	4	5	6	7	8	9	10
B	11	12	13	14	15	16	17	18	19	20	21	22
C	23	24	25	26	27	28	29	30	31	32	36	37
D	39	40	41	43	44	45	46	47	48	49	50	51
E	52	53	54	55	56	57	58	59	60	61	62	63
F	64	65	66	67	68	69	MSE	PIG				plas.


1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F

Richards, 1982), as the hybridization probe, and human genomic DNA as the positive control. This probe detects only the hMT-IIA gene in the human genome, and none of the related human MT genes (McInnes *et al.*, 1987a).

ii) Slot-blot analysis

The number of copies of the pHMPG.4 insert present in each of the transgenic mice was determined by slot-blot analysis (6.3.2.iv). Five ug samples of DNA from each of the available tail samples was denatured and applied to Gene-screen hybridization membrane through a slot-blot apparatus. Samples of denatured pHMPG.4 DNA corresponding to genomic copy numbers ranging from 1 to 80 copies per cell, combined with 5 ug of denatured mouse control DNA (as carrier), were applied to the same membrane. This slot-blot was hybridized to the nick-translated *HindIII*/*AvaI* hMT-IIA promoter insert, followed by washing at high stringency (6.3.2.iv), and is illustrated in Figure 4-5. Comparison of the intensity of hybridization of genomic samples to those of the plasmid standards was performed using a Zeineh laser densitometer, and indicated that the number of copies of the pHMPG.4 sequence present in different mice ranged from one to greater than one hundred copies per cell (Table 4-1). The number of copies present in mice not analysed by slot-blot (due to shortage of sample) were estimated by comparing their intensity of hybridization on the original dot-blot (Figure 4-4) to that of mice with more accurately determined copy numbers.

The range of copy numbers, from 1 to 150, and the frequency of integration for the pHMPG.4 mice is similar to that observed by others (see references in Sections 1.4 and 4.1).

4.3.3 Growth rates of transgenic mice

The growth rates of transgenic mice, and their non-transgenic (control) littermates, were determined by weighing each animal at weekly intervals. Many of the transgenic mice were clearly larger than their litter-

FIGURE 4-5

Slot-blot analysis of pHMPG.4 F0 transgenic mice

5 ug samples of denatured transgenic mouse DNA were applied to a membrane along with mouse (MSE) and human (HUM) negative and positive controls (4.3.2.ii). Various amounts of pHMPG.4 plasmid DNA were combined with 5 ug of control mouse DNA and applied to the same filter. The amounts of pHMPG.4 plasmid corresponded to gene copy numbers of between 1 and 80 copies of homologous sequence per cell, adjusted for the proportion of the plasmid homologous to the hybridization probe (one fifth) and assuming a mouse diploid genome size of 5 pg (Sober, 1970). As the 2 copies/cell plasmid dot is of equal intensity as the human DNA control, these calculations can be taken as being reasonably accurate. The probe used was the nick-translated *Hind*III/*Ava*I hMT-IIA promoter fragment isolated from pUCMT (Figure 4-1). A key indicating the indentity of each sample is illustrated below. The number in brackets indicates the number of copies per cell equivalent to the amount of pHMPG.4 plasmid DNA.

	A	B	C	
1	4	36	54	
2	5	40	57	
3	8	45	62	
4	26	50	65	
5	28	51	69	
6	29	MSE	HUM	
7				
8	1000 (80)	300 (24)	50 (4)	
9	500 (40)	200 (16)	25 (2)	pg pHMPG.4
10	400 (32)	100 (8)	12.5 (1)	

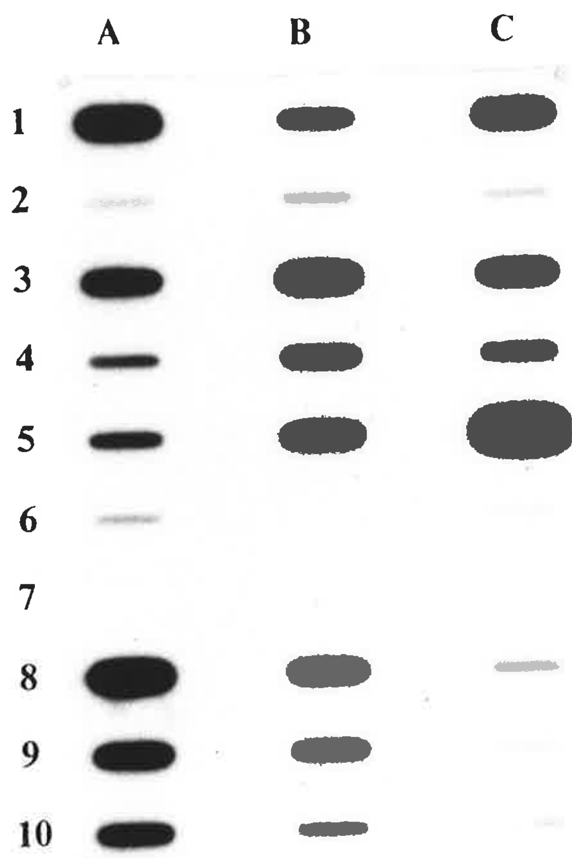

A
B
C
1
2
3
4
5
6
7
8
9
10

TABLE 4-1

F0 pHMPG.4 transgenic mice

The data presented in this Table illustrates the sex, number of copies of the foreign gene per cell, and growth ratio (body weight divided by the mean body weight of sex-matched non-transgenic control littermates) at approximately 12 weeks of age of each of the F0 pHMPG.4 transgenic mice (4.3.2; 4.3.3).

Animal no.	sex	pHMPG.4 gene copy no.	growth ratio
4	M	70	1.32
5	F	3	0.99
8	F	36	1.34
20	F	1	1.91
25	F	10	1.27
26	F	8	1.63
28	M	14	N.D.
29	F	3	1.56
36	M	14	0.98
40	M	4	1.57
45	M	42	1.39
49	F	15	0.96
50	F	16	1.12
51	F	36	N.D.
54	M	36	1.25
57	M	3	0.77
62	F	32	1.15
65	M	10	1.69
67	M	15	0.89
69	F	150	1.91

mates by 5 weeks of age. This difference became greater with time, and by 12 weeks of age some of the transgenic mice were almost twice the size of the controls. The size difference between the animals is illustrated in Figure 4-6, which shows one of the transgenic females alongside one of her non-transgenic sisters. The growth ratios (body weight divided by the mean body weight of sex-matched littermates of the same age) of these mice are listed in Table 4-1, and illustrated in chart form, according to sex, in Figure 4-7. These results indicate that most of the transgenics, 8 of the 10 surviving females (80 %) and 5 of the 8 surviving males (62 %), grew faster than their control littermates, the largest animals being almost twice the average size of their non-transgenic littermates.

The pHMPG.4 construct therefore appears to be expressed at a level sufficient to promote growth in a proportion (72 %) of the transgenic animals. The increased growth rates observed are similar to those in transgenic mice containing copies of MT-I/ rat or human GH fusion genes, which had growth ratios ranging from 0.87 to 2.37 (Palmiter _et al._, 1982b, 1983). Mice containing the pHMPG.4 construct with growth ratios of up to 2.35 are discussed below (4.3.4).

The growth ratio does not appear to be directly correlated with the number of copies of the foreign gene present in transgenic mice (Table 4-1). This is not surprising as similar results have been obtained by others studying the expression of human GH and GHRF in transgenic mice (Palmiter _et al._, 1983; Hammer _et al._, 1984, 1985b; Morello _et al._, 1986).

4.3.4 Fertility of transgenic mice, and inheritance of the fusion gene

The ability of the pHMPG.4 transgenic animals to produce offspring was determined by mating a number of animals with CBAxC57 F1 control animals. The ability of an animal to produce offspring is shown in Table 4-2. Each of the males, with one exception, sired at least one litter. The male which

FIGURE 4-6

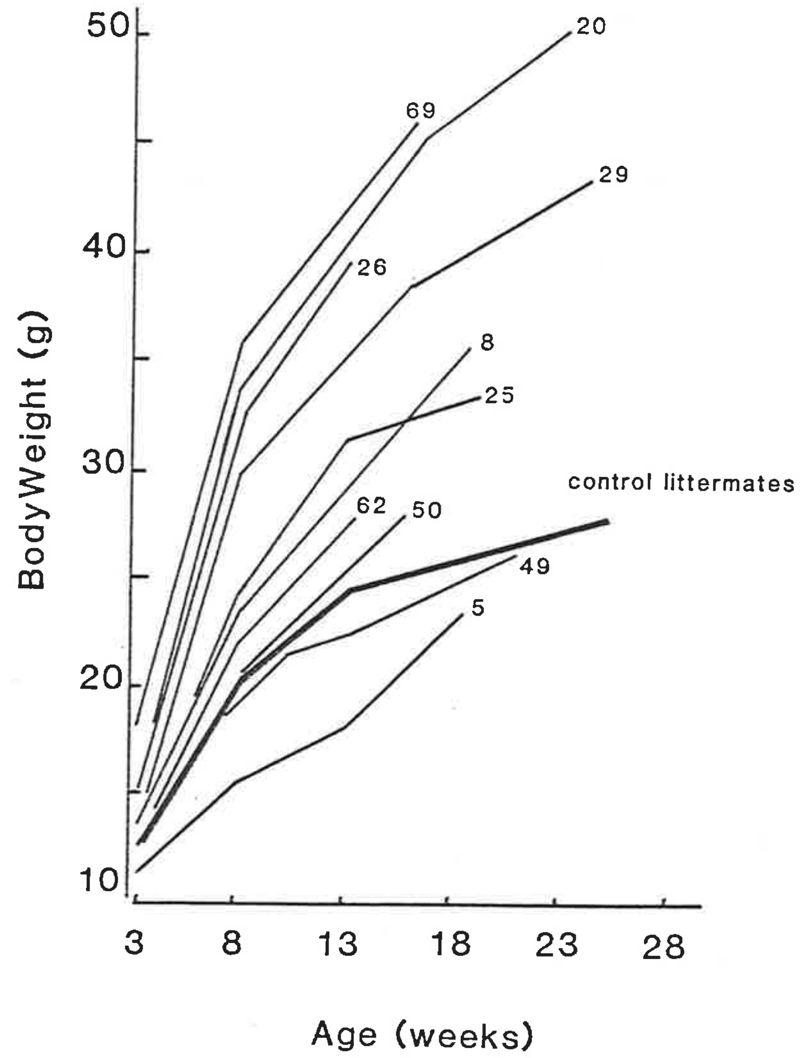
Growth of transgenic versus non-transgenic mice

The size of two female littermates at around 12 weeks of age are illustrated (4.3.3). The darker coloured mouse on the left is not transgenic, while the lighter coloured mouse on the right is transgenic mouse #20. The transgenic mouse (which contains only one copy of the pHMPG.4 insert per cell) has a growth rate 1.91 times that of her non-transgenic littermates (Table 4-1).

FIGURE 4-7

Growth of pHMPG.4 F0 transgenic mice

The growth rates of each of the transgenic mice is compared with the average size of their sex matched non-transgenic control littermates (4.3.3). Eight out of ten females (80 %), and five out of eight transgenic males (62 %) are growing faster than their control littermates. The growth rates of each of these animals is presented in Table 4-1.


Females
50
40
30
20
10
BodyWeight (g)
3
8
13
18
23
28
Age (weeks)
20
69
29
26
8
25
control littermates
62
50
49
5

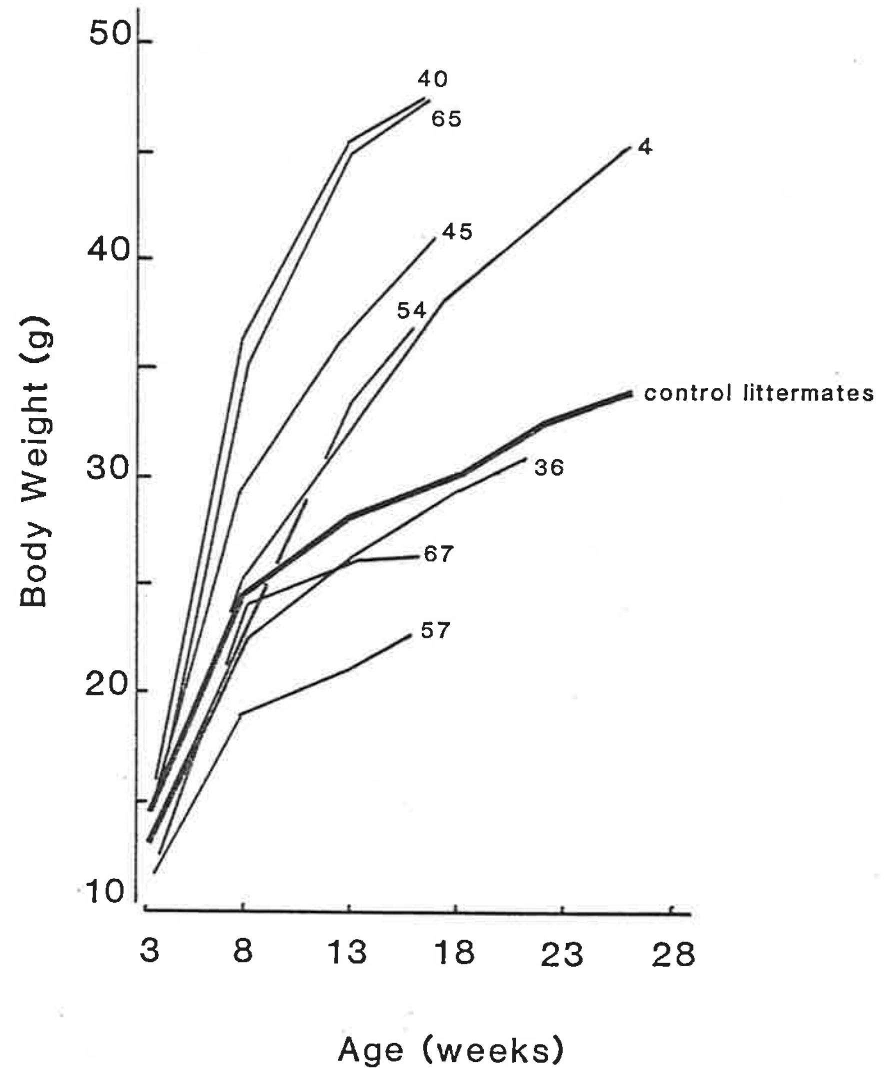

Males
50
40
30
20
10
Body Weight (g)
3
8
13
18
23
28
Age (weeks)
40
65
4
45
54
control littermates
36
67
57

TABLE 4-2

The growth rate and fertility of pHMPG.4 F0 mice, and the transmission of the foreign genes to F1 animals

This Table illustrates the association between enhanced growth rates and infertility in transgenic female mice (4.3.4). These results indicate that the expression of PGH in male mice has no effect on fertility. This Table also illustrates the proportion of offspring to which each of these transgenic mice can pass on their foreign genes.

MALES

Animal no.	Growth ratio	Offspring	transmission
65	1.69	+	22 %
40	1.57	+	37 %
45	1.39	+	36 %
4	1.32	+	55 %
54	1.25	+	48 %
36	0.98	-	
67	0.89	+	25 %
57	0.77	+	7 %

FEMALES

Animal no.	Growth ratio	Offspring	transmission
69	1.91	+	0 % (0/3)
20	1.91	-	
26	1.63	-	
29	1.56	-	
8	1.34	-	
25	1.27	-	
62	1.15	+	0 % (0/6)
50	1.12	+	21 %
5	0.99	+	31 %
51	0.97	+	0 % (0/5)

produced no offspring appeared to have some behavioural, rather than physiological abnormality. The transgenic females however were found to produce very few offspring if their growth ratios were greater than 1.15 (Table 4-2). Only one female (#69) with a high growth ratio (1.91) was capable of producing any offspring, and in this case only a single, very small litter (3 pups) was produced.

The offspring of a number of the transgenic mice were analysed to determine if they had inherited the foreign gene. Dot-blots were prepared which contained DNA samples from the progeny of twelve of the transgenic mice along with samples of the parental DNAs and the appropriate positive and negative controls (6.3.2.iv). These dot-blots were hybridized to the nick-translated (6.3.5.i) hMT-IIA promoter HindIII/AvaI probe (Figure 4-8).

Of the twelve F0 (founder) mice analysed, nine (75 %) passed on the hMT-IIA/PGH fusion gene to at least a portion of their offspring (F1, first generation). The frequency of transmission of the fusion gene for each of these mice is illustrated in Table 4-2. The percentage of offspring from any one parent containing the foreign gene varies from 0 (three mice) to 55 % (mouse #4). The low frequency of transmission observed for a number of mice, less than 35 %, is a result consistent with these mice being germ line mosaics (Wagner et al., 1983; Palmiter et al., 1984; Soriano and Jaenisch, 1986), although in some cases it may simply be due to the small sample size. The intensity of hybridization also varies markedly between some of the parents and different members of their offspring, indicating that either these F0 mice are also mosaics, or that they contain the foreign gene sequences inserted into multiple chromosomal sites. If the latter case is correct, these mice may still be mosaics as less than 50 % of the offspring inherit each of the different loci. The clearest example of variation in copy number can be seen in the progeny of mouse #4, which

FIGURE 4-8

Inheritance of foreign genes in the offspring of pHMPG.4 founder mice

Dot-blots demonstrating the transmission of the pHMPG.4 genes in F0 to F1 mice are illustrated (4.3.4). 5 ug samples of DNA from each mouse, along with controls, was denatured and filtered onto a membrane (6.3.2.iv). This membrane was hybridized to the nick-translated (6.3.5) hMT-IIA HindIII/ AvaI insert isolated from pUCMT (Figure 4-1). DNA from each of the F0 parents is included alongside that of their progeny. A key indicating the identity of each of the samples on these dot-blots is shown below (asterisks indicate F0 parents). Arrows indicate some of the fainter positive dots.

a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	*4	4-1	4-2	4-3	4-4	4-5	4-6	4-7	4-8	4-9	4-10	4-11
B	4-12	4-13	4-14	4-15	4-16	4-17	4-18	4-19	4-20	4-21	4-22	4-23
C	4-25	4-26	4-27	4-28	4-29	4-30	4-31	4-32	4-33	4-34		
D	*5	5-1	5-2	5-3	5-4	5-5	5-6	5-7	5-8	5-8A	5-8B	5-10
E	5-11	5-12								PIG	MSE	HUM

b)

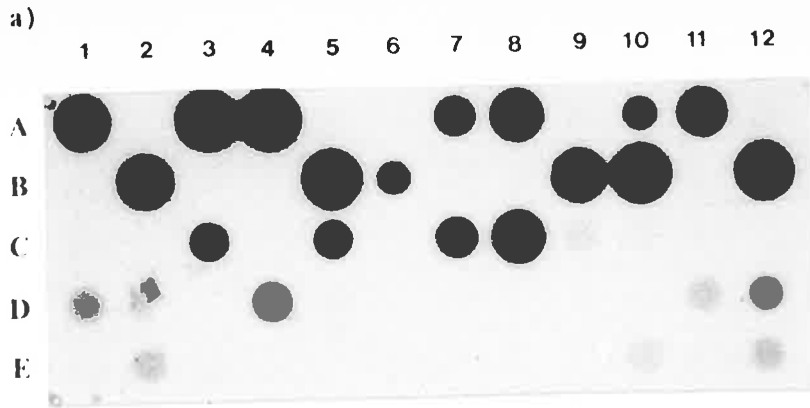
	1	2	3	4	5	6	7	8	9	10	11	12
A	*40	40-1	40-2	40-3	40-4	40-5	40-6	40-7	40-8			
B	*50	50-1	50-2	50-3	50-4	50-5	50-6	50-7	50-8	50-9	50-10	50-11
C	50-12	50-13	50-14	50-15	50-16	50-17	50-18	50-19				

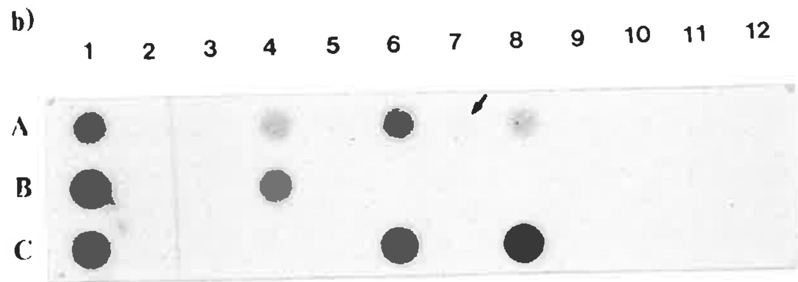
c)

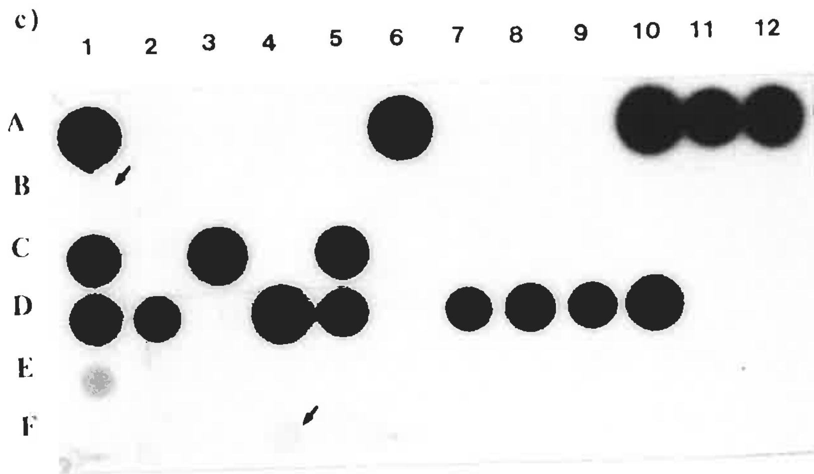
	1	2	3	4	5	6	7	8	9	10	11	12
A	*45	45-1	45-2	45-3	45-4	45-6	45-7	45-8	45-9	45-10	45-11	45-12
B	45-13	45-14										
C	*54	54-1	54-2	54-3	54-4	54-5	54-6	54-7	54-8	54-9	54-10	54-11
D	54-12	54-13	54-14	54-15	54-16	54-17	54-18	54-19	54-20	54-21		
E	*57	57-0	57-1	57-2	57-3	57-4	57-5	57-6	57-7	57-8	57-9	57-11
F	57-12	57-13	57-14	57-15								

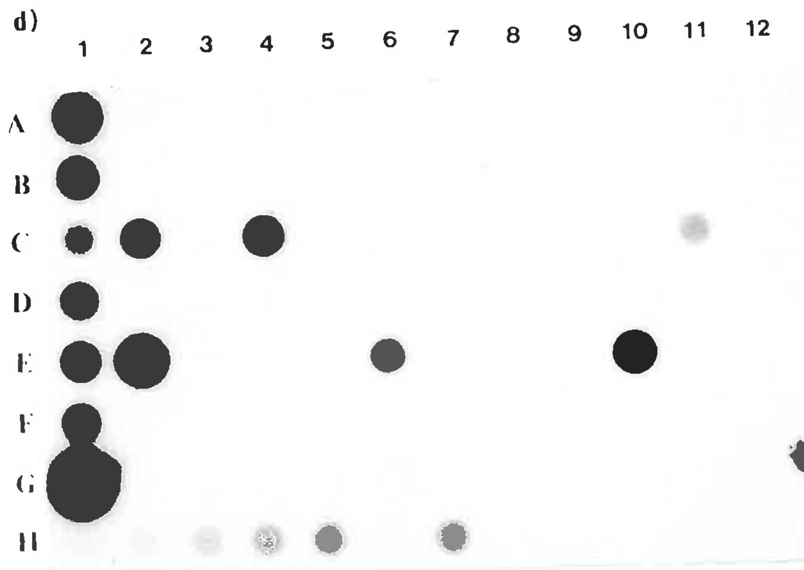
d)

	1	2	3	4	5	6	7	8	9	10	11	12
A	*51	51-1	51-2	51-3	51-4	51-5						
B	*62	62-1	62-2	62-3	62-4	62-5	62-6					
C	*65	65-1E	65-2E	65-3E	65-4E	65-5	65-6	65-7	65-8	65-9	65-10	65-11
D	65-12	65-13	65-14	65-15	65-16	65-17	65-18					
E	*67	67-1E	67-2E	67-3	67-4	67-5	67-6	67-7	67-8	67-9	67-10	67-11
F	67-12	67-13	67-14	67-15	67-16							
G	*69	69-1	69-2	69-3								
H	5pg	10pg	20pg	30pg	40pg	MSE	HUM					


a)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E


b)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C


c)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F


d)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

differ in hybridization intensity by greater than a factor of ten (Figure 4-8). This result indicates that even the animals which transmit the fusion gene to one half of their offspring (e.g. #4), may in some cases still be mosaics. Pedigrees which demonstrate the inheritance pattern of these mice are shown in Figure 4-9.

The transgenic offspring of the pHMPG.4 mice often inherited the large growth phenotype of their transgenic parent. In Table 4-3 the growth ratio of the transgenic offspring is compared to that of their parent. Although many of the transgenic offspring are growing much larger than their control littermates, the growth rates of transgenic mice sired by the same parent are highly variable. For example, growth ratios of the male transgenic offspring of mouse #54 (growth ratio 1.25) vary from 1.18 to 2.35.

4.4 Transgenic pigs

4.4.1 Production of transgenic pigs

The *HindIII/PvuI* fragment of pHMPG.4 which had been demonstrated to integrate at a high frequency and be efficiently expressed in transgenic mice (4.3.2, 4.3.3) was used to produce transgenic pigs. Single cell *in vivo* fertilized pig embryos were collected from superovulated large white sows. These were prepared and injected with approximately 600 copies of the pHMPG.4 insert (6.3.11.ii). Approximately 30 injected embryos were surgically transferred into the oviducts of each of a number of synchronized recipient sows. Four of these sows farrowed small litters (4-5 per litter), producing a total of 17 piglets.

4.4.2 Analysis of transgenic pigs

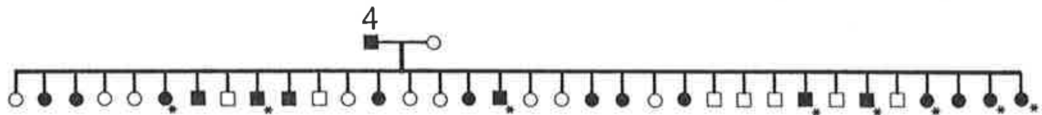
i) Dot-blots

The piglets were tested for the presence of the foreign gene by dot-blot hybridization (6.3.2.iv) of DNA isolated from tail tissue (6.3.1.ii).

FIGURE 4-9

Transmission of the foreign gene to F1 and F2 mice

The F1 data presented in the dot-blots in Figure 4-8 is shown here in pedigree form (4.3.4). Circles represent females and squares males. Diamonds represent foetuses of unknown sex. Solid and open colouring indicates if mice are transgenic or non-transgenic respectively. The variation in foreign gene copy number seen in some of the F1 mice (4.3.4) is shown here by marking mice with a copy number lower than their parents with an asterisk, and by marking mice with a copy number much higher than their parents with a triangle. Each of the F2 mice studied contained an equal number of copies of the foreign gene as their F1 parent.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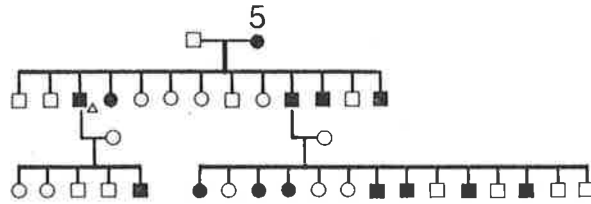
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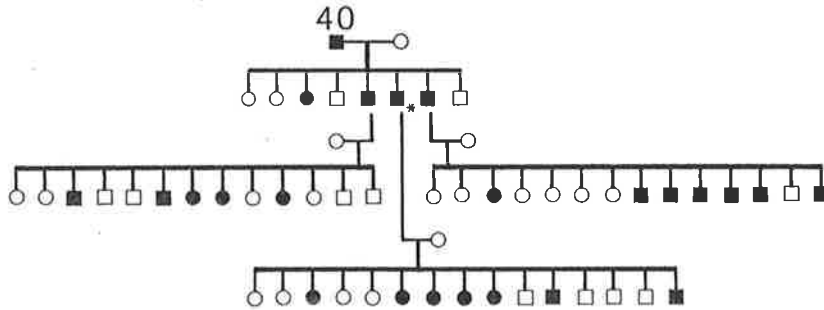
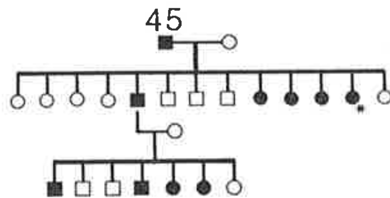
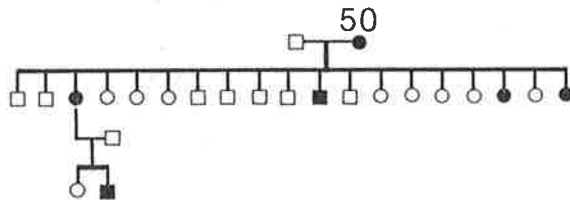
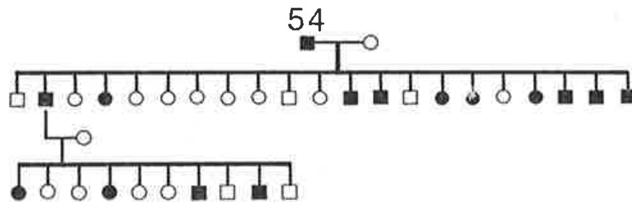
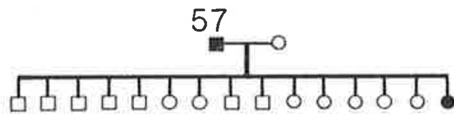
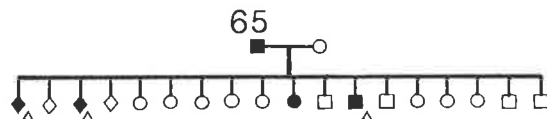

40


45


50


54


57


65

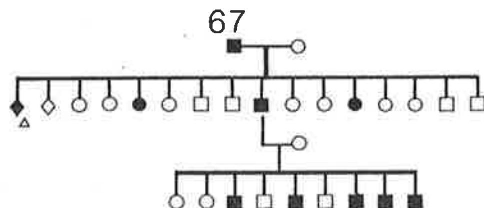

67

TABLE 4-3

Growth rate of F0 versus F1 pHMPG.4 transgenic mice

This Table illustrates the growth ratios of each of the pHMPG.4 F0 and F1 mice (at 20 weeks of age). The mean growth ratio of the offspring of each family according to sex are also indicated. The offspring of mouse #4 which contain a high number of copies of the foreign gene (4.3.4) are indicated with an asterisk (Figure 4-8). The offspring of mouse #45 which contains many fewer copies of the foreign gene than their transgenic parent is indicated with a square (4.3.4).

FO parent (no.)	Sex	growth ratio	F1 Males	$\overline{x}$	F1 Females	$\overline{x}$
4	M	1.32	0.89* 0.86 1.29* 1.01 0.87	0.98	1.23* 1.14 1.09* 1.04* 1.23* 1.36* 1.59* 1.10 1.19* 1.05	1.20
5	F	0.99	1.21 0.78 0.78	0.92	–	–
40	M	1.57	1.84 1.90 1.51	1.75	2.18	2.18
45	M	1.39	1.45	1.45	1.02 0.89 1.01 1.52□	1.11
50	F	1.12	–	–	1.15	1.15
54	M	1.25	2.35 1.45 1.18 1.81 1.51 1.53	1.64	1.47 1.38	1.43
65	M	1.69	1.45	1.45	1.57	1.57
67	M	0.89	1.66	1.66	1.58 1.57	1.58

Both normal pig DNA and human genomic DNA were included on these dot-blots to act as negative and positive controls respectively. Following the hybridization of these dot-blots to the HindIII/AvaI fragment of the hMT-IIA promoter a number of positive signals were evident. Four of the pigs showed strong hybridization equivalent to greater than one copy per cell and a further two showed weak hybridization, slightly above background, and equivalent to less than one copy per cell (Figure 4-10).

ii) Slot-blots

The number of copies per cell of the pHMPG.4 insert present in each of the transgenic pigs was determined by slot-blot analysis. Five ug samples of tail DNA from each of the transgenic animals was filtered onto a slot-blot along with human and pig positive and negative controls (6.3.2.iv). A range of amounts of pHMPG.4 plasmid DNA (which also contained 5 ug of control pig genomic DNA) corresponding to genomic copy numbers equivalent to between one and fourty copies per cell were also included. This slot-blot was hybridized to the nick-translated HindIII/AvaI fragment of the hMT-IIA promoter (4.3.2) and washed at high stringency (6.3.2.iv). The intensity of hybridization of the transgenic animals was compared to the plasmid standards by laser densitometry, and found to range from approximately 0.5 copies per cell in animals #375 and #739 to 15 copies per cell in animal #295 (Table 4-4; Figure 4-11).

iii) Southern Analysis

The organization of the foreign sequences within the transgenic pigs was studied by Southern blotting (6.3.2.iv). There are no BamHI sites within the pHMPG.4 insert (Figure 4-3). The digestion of the genomic DNA of the transgenic animals with this enzyme should therefore produce bands on genomic Southerns, the length of which are governed by the distance of the nearest BamHI sites to the site of integration, the number of integration

FIGURE 4-10

Analysis of potentially transgenic pigs by dot-blot

10, 5, and 1 ug samples of DNA isolated from the tails of pigs (6.3.1.ii) which had developed from eggs microinjected with the insert of pHMPG.4, were denatured and applied to a membrane (4.4.2.i). Plasmid pHMPG.4 and human (HUM) genomic DNA positive controls, and pig (PIG) genomic DNA negative controls were included on each dot-blot. The plasmid positive controls are in row 1, blot A. In this row the numbers in brackets refer to the number of copies per cell each plasmid dot is equivalent to. Samples in rows A4 to A11 and B1 to B9 contain the test pig samples. The nick-translated hMT-IIA promoter HindIII/ AvaI insert was used as the hybridization probe. A key indicating the identity of the sample on each dot is shown below.

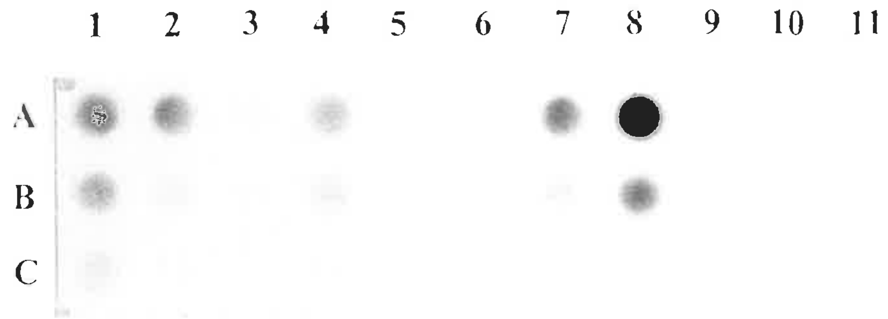
A.

	1	2	3	4	5	6	7	8	9	10	11
A. 10ug	(2)	HUM	PIG	177	178	179	180	295	296	297	298
B. 5ug	(1)	"	"	"	"	"	"	"	"	"	"
C. 1ug	(0.5)	"	"	"	"	"	"	"	"	"	"

B.

	1	2	3	4	5	6	7	8	9	10	11
A. 10ug	373	374	375	376	735	736	737	738	739	PIG	HUM
B. 5ug	"	"	"	"	"	"	"	"	"	"	"
C. 1ug	"	"	"	"	"	"	"	"	"	"	"

A



B

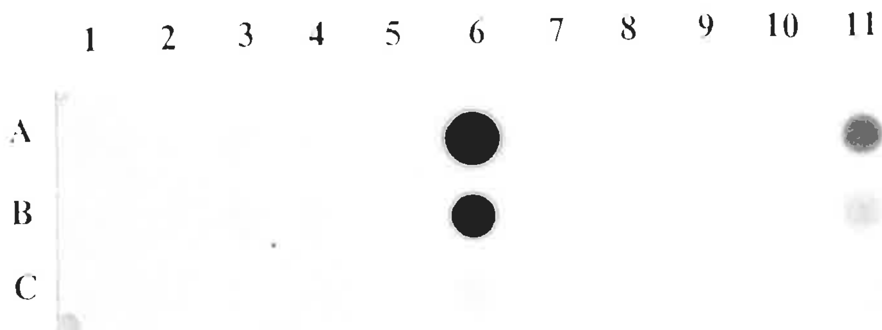
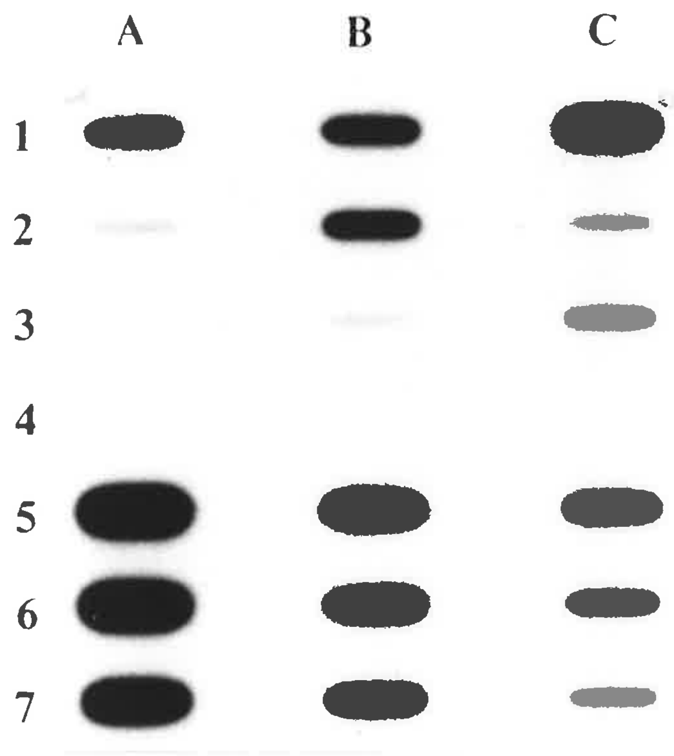


FIGURE 4-11

Slot-blot analysis of transgenic pigs

Samples (5 ug) of transgenic pig DNA and pig (PIG) and human (HUM) negative and positive controls were denatured and applied to a membrane along with a number of samples containing various amounts of pHMPG.4 plasmid DNA combined with 5 ug of pig control DNA. The amounts of pHMPG.4 DNA applied correspond to gene copy numbers of between 1 and 40 gene copies per cell (shown in brackets below) assuming similar circumstances to those in Figure 4-5, and a porcine genome size of 5 pg/cell (Sober, 1970). The probe used was the nick-translated hMT-IIA promoter HindIII/AvaI insert. A key to the samples on the blot is given below. Quantitation of the intensity of hybridization of each of the samples on this membrane was performed using a laser densitometer. The results of this analysis are presented in Table 4-4.

	A	B	C	
1.	177	180	295	
2.	375	736	739	
3.		PIG	HUM	
4.				
5.	500 (40)	125 (10)	50 (4)	
6.	375 (30)	100 (8)	25 (2)	pg pHMPG.4 (copy no.)
7.	250 (20)	75 (6)	12.5 (1)	



A
B
C
1
2
3
4
5
6
7

TABLE 4-4

Transgenic pigs: gene copy number, growth, and serum PGH concentration

The number of copies (per cell) of the foreign gene present in each of the transgenic pigs estimated by slot-blot analysis (4.4.2.ii), the daily weight gain (between days 50 and 120) and the serum PGH concentration of each of the transgenic pigs and their non-transgenic littermates are illustrated. The female control values are the mean of three animals, and the male control values are the mean of two animals.

Pig no.	sex	copy no./cell	daily weight gain (gm)	serum PGH conc. (ng/ml)
177	F	3	765	10.4
295	F	15	953	27.8
739	F	0.5	686	6.9
controls	F	-	806	10.6
180	M	6	851	15.3
375	M	0.5	487	6.3
736	M	6	857	11.1
controls	M	-	670	15.3

sites, and the number of integrated copies of the pHMPG.4 insert.

BamHI digests of genomic DNA from each of the pigs was fractionated by agarose gel electrophoresis and transferred to Zeta-probe hybridization membranes as described (6.3.2.iv). These membranes were then hybridized to the nick-translated HindIII/AvaI insert of the hMT-IIA promoter (4.3.2). Autoradiographs of these blots confirmed earlier results by demonstrating hybridization to bands in tracks containing DNA from all but one of the pigs identified as transgenic by dot-blot analysis. BamHI digests of DNA from transgenic pigs with greater than one copy per cell produced a single band which hybridized to the hMT-IIA probe (Figure 4-12). Of the two low copy number pigs (less than one per cell), bands were visible only in pig #375 DNA after extremely long exposure (160 hour), even though the single copy human MT-IIA gene positive control was visible on much shorter (20 hour) exposures. Interestingly, this animal produced three BamHI bands which hybridized to the hMT-IIA probe (Figure 4-12), indicating that this animal contains three very small fragments of the hMT-IIA promoter at three separate chromosomal locations.

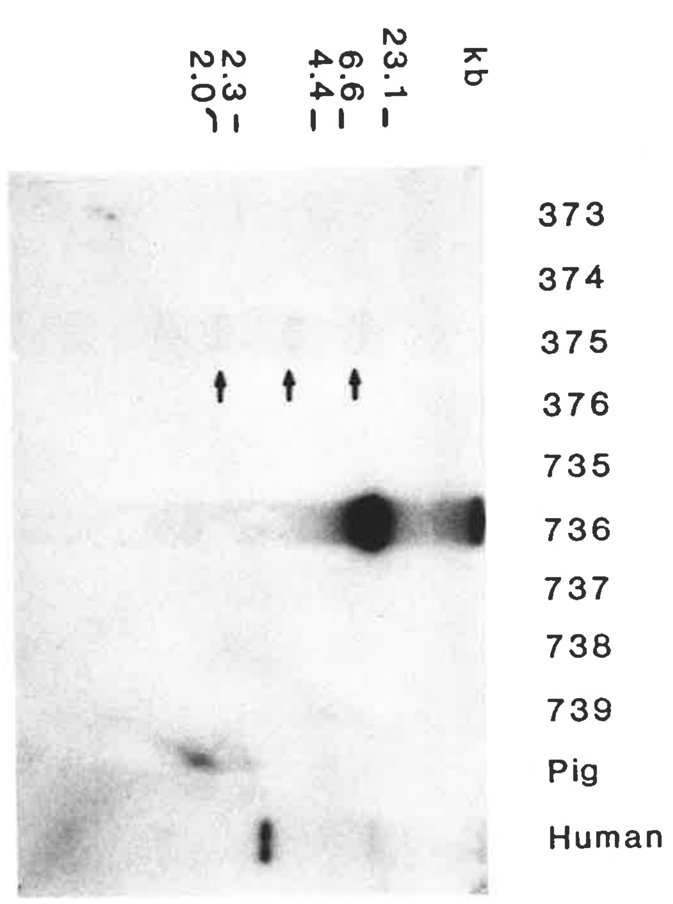
The pattern of integration was studied by performing Southern blots of EcoRI digested DNA from the transgenic pigs, which were probed with the hMT-IIA promoter probe. EcoRI cuts twice within the pHMPG.4 plasmid insert (Figure 4-3), and restricting with this enzyme followed by probing with the hMT-IIA promoter allows the detection of flanking EcoRI sites, head to head multimers and head to tail multimers.

The autoradiographs of Southern blots containing EcoRI restricted DNA from each of the transgenic pigs are illustrated in Figure 4-13. These results fail to detect the normal pig DNA control (and the low copy number transgenic pigs #375 and #739) but clearly detect the single copy number human MT-IIA gene positive control. Head to head integration of the pHMPG.4

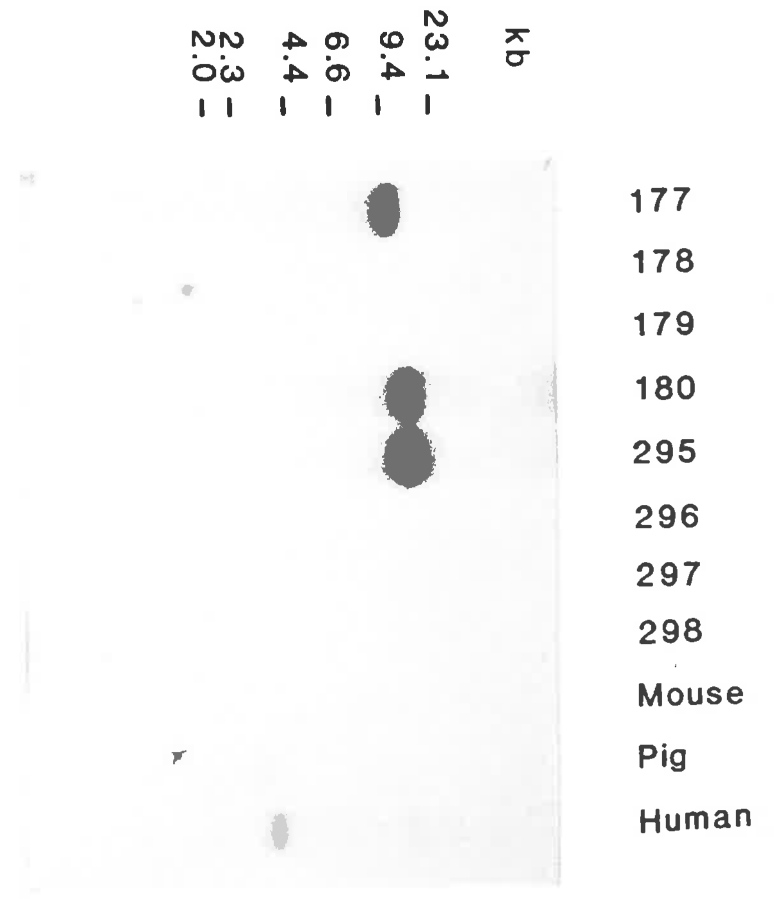
FIGURE 4-12

Genomic Southerns of BamHI digested transgenic pig DNA

A and B: Southern blots containing BamHI digested transgenic pig DNA (samples 177 to 298 and 373 to 739; 4.4.2.iii). 3 ug samples of DNA from pigs which developed from microinjected eggs, along with human positive and pig and mouse negative controls, was digested with BamHI, electrophoresed, and transferred to Zetaprobe membranes (6.3.2.iv). These membranes were hybridized to the nick-translated hMT-IIA promoter HindIII/AvaI insert, followed by washing in 0.1 X SSC/0.1 % SDS at 65 ^{o}C. The exposure time of autoradiograph B was five times that of A, due to the extremely faint nature of the three bands present in pig #375 DNA, which have been highlighted with arrows.



kb
23.1
6.6
4.4
2.3
2.0
373
374
375
376
735
736
737
738
739
Pig
Human



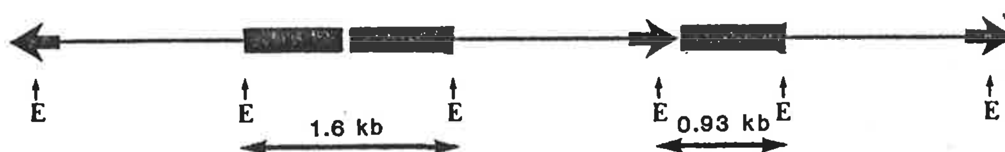
kb
23.1
9.4
6.6
4.4
2.3
2.0
177
178
179
180
295
296
297
298
Mouse
Pig
Human

FIGURE 4-13

Genomic Southern analysis of EcoRI digested transgenic pig DNA

Southern blots (6.3.2.iv) containing EcoRI digested (+) and undigested (-) samples from each of the transgenic pigs (4.4.2) plus human positive and pig negative controls (4.4.2.iii). These blots were hybridized to the nick-translated hMT-IIA promoter HindIII/AvaI insert, washed at high stringency (0.1 X SSC/ 0.1 % SDS at 65 °C), and autoradiographed. The position of the molecular weight markers which were included on each gel are indicated.

The expected EcoRI restriction pattern resulting from either "head to head" or "head to tail" integration events, detected with a hMT-IIA promoter probe are indicated below. The solid areas represent the hMT-IIA promoter region, which is detected by the hybridization probe. The arrows indicate transcriptional orientation.



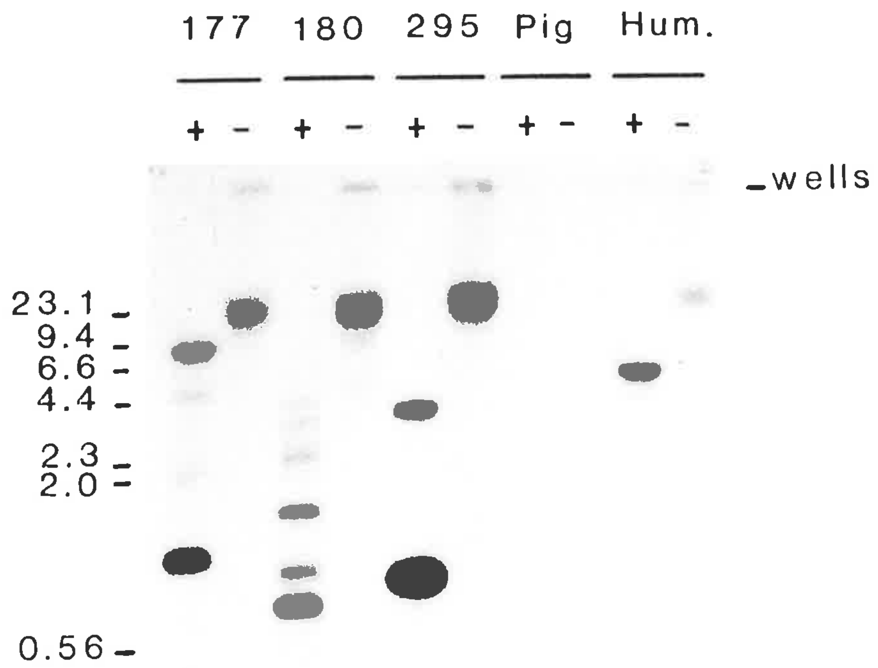
The size of the bands detected (in kb) are:

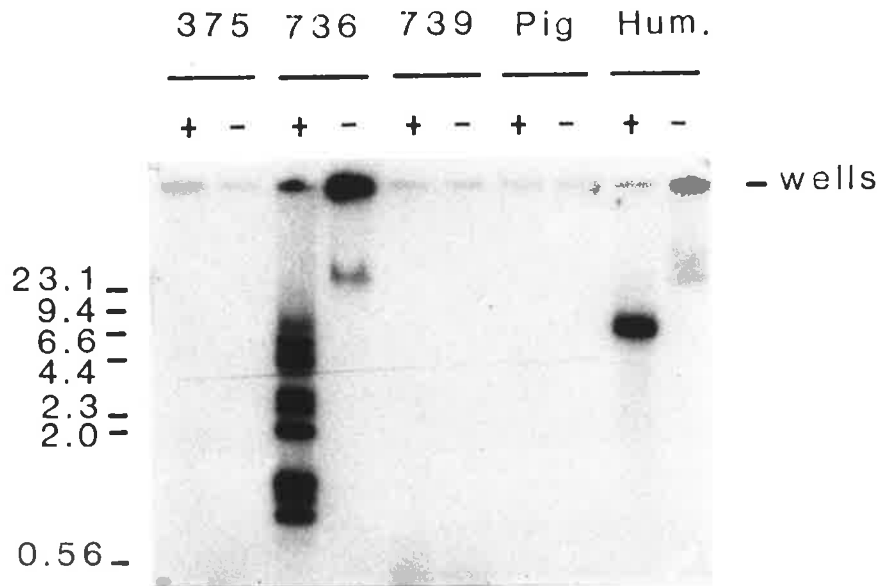
177; 6.6, 1.24

180; 2.5, 1.68, 1.03, 0.79

295; 4.0, 0.93

736; 7.0, 6.5, 5.3, 3.9, 3.6, 2.75, 2.55, 2.3, 1.9, 1.35, 1.16, 1.0


177
180
295
Pig
Hum.
+ - + - + - + - + -
wells
23.1
9.4
6.6
4.4
2.3
2.0
0.56


375
736
739
Pig
Hum.
+ - + - + - + - + -
wells
23.1
9.4
6.6
4.4
2.3
2.0
0.56

insert should produce an EcoRI band of 1.6 kb, head to tail integration a band of 930 bp, and in some cases, additional bands generated by the EcoRI sites flanking the integration site (Figure 4-13). Of the four high copy number pigs, only pig #295 produces an easily interpretable banding pattern, with a bright band at 930 bp and a single fainter band indicating that all 15 copies of the pHMPG.4 insert are integrated in a head to tail fashion in this animal. Pigs #180 and #736 each produce a large number of bands (Figure 4-13), none of which correspond to head to tail integration, and only one of which in #180 may correspond to a head to head configuration. As in each of these animals the multple copies of the fusion gene have integrated into single chromosomal sites (Figure 4-12), the organization appears to be in a random pattern, and, because of the large number of bands, may include a number of rearranged sequences. This seems especially likely in pig #736, which has many more EcoRI bands than it has gene copies.

4.4.3 Growth of transgenic pigs

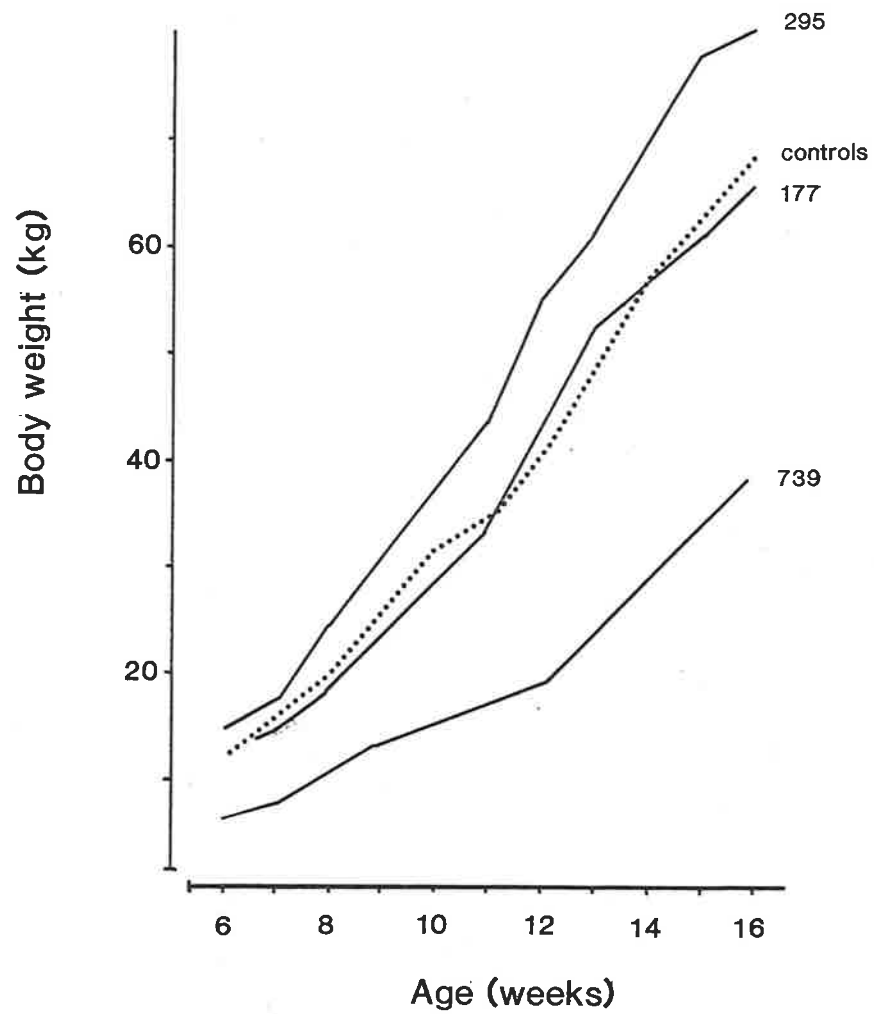
The growth of transgenic pigs was monitored by weighing animals at weekly intervals, and is illustrated in Figure 4-14. Transgenic pig #295 was found to be growing at a significantly greater rate than her non-transgenic littermates. This difference in growth rate is demonstrated clearly by comparing the daily weight gain over a ten week period from 50 to 120 days after birth. Over this period pig #295 grew at 953 g/day versus female controls which grew at 806 g/day, an increase of 15.4 % in daily weight gain (Table 4-4). This resulted in an animal which was 1.18 times the size of her control littermates at 16 weeks of age.

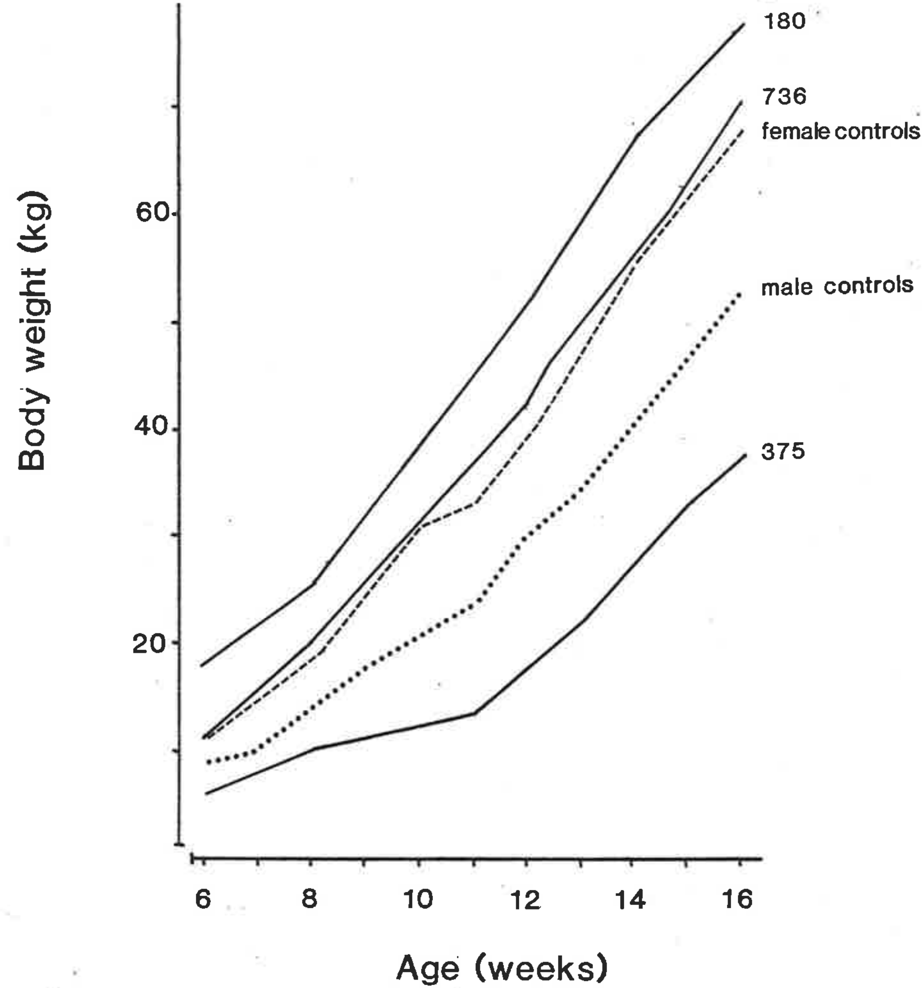
The serum PGH content of these animals was determined by S.Dean (Department of Obstetrics and Gynaecology, University of Adelaide) using an enzyme linked double antibody detection system. Serum PGH levels were

FIGURE 4-14

Growth rate of transgenic pigs

The growth performance of each of the transgenic pigs is plotted against the growth rate of non-transgenic littermates (4.4.3). The dotted lines represent the average growth rate of sex-matched non-transgenic littermates, the dashed line represents the average of non-transgenic littermates of the opposite sex, and solid lines represent transgenic animals.


Females
Body weight (kg)
60
40
20
295
controls
177
739
6
8
10
12
14
16
Age (weeks)


Males
Body weight (kg)
60
40
20
180
736
female controls
male controls
375
6
8
10
12
14
16
Age (weeks)

similar to, or below control levels in transgenic pigs #177, 736, 375 and 739. The transgenic male #180 had a slightly higher than average concentration (15.3 versus 12.0 ng/ml), and the rapidly growing female #295 had a serum PGH level 2.6 times that of the female controls (Table 4-4).

Unfortunately pig #295 contracted a lung infection at 18 weeks of age, lost weight rapidly, and died approximately 10 days later. The transgenic male #180 was in severe pain due to the development of arthritis, and therefore sacrificed at 25 weeks of age.

4.4.4 Expression of PGH mRNA in transgenic pig number 180

The death of pig #295 occurred during the night and no fresh tissue samples were available for RNA analysis. The death of pig #180 however provided an opportunity to examine the expression pattern of PGH mRNA to determine where, and if, the hMT-IIA/PGH fusion gene was active in this animal.

Total RNA was isolated from six tissues, the liver, kidney, spleen, brain, pituitary and testis. These tissues were chosen as representative of the tissues found to express HGH mRNA in transgenic mice containing integrated MT-I/HGH (Palmiter *et al.*, 1983) and hMT-IIA/HGH (Hammer *et al.*, 1985a) fusion genes and transgenic rabbits containing MT-I/HGH or hMT-IIA/HGH fusion genes (Hammer *et al.*, 1985a, 1985c). The pituitary RNA was isolated for use as a positive control. Ten ug of total RNA from each of these tissues was denatured and fractionated on a 1.5 % agarose gel (6.3.9.iii), along with 10 ug of liver RNA isolated from a non-transgenic pig. Following electrophoresis the RNA was transferred to a Gene-screen hybridization membrane (6.3.9). This membrane was hybridized (6.3.8.iii) to a 26 base long oligonucleotide, MT.26, which is homologous to bases +35 to +60 of the hMT-IIA 5' untranslated region (Figure 4-2), which had been kinased (6.3.5.ii) to a specific activity of 5×10^8 cpm/ug. Following a low

stringency wash (6 x SSC at 65 ^{o}C) the filter was autoradiographed. Inspection of the resulting autoradiogram failed to identify any bands of hybridization corresponding to transcripts from the fusion gene.

The Northern blot was then washed at high stringency, followed by hybridization to plasmid pPG.3, a full length PGH cDNA clone (2.2), which had been radioactively labelled by nick-translation to a specific activity of 2 X 10^{9} cpm/ug (6.3.5.i). This filter was washed at high stringency (0.1 X SSC/ 0.1 % SDS at 65 ^{o}C) and autoradiographed.

Examination of the Northern hybridized to the PGH cDNA probe revealed a band of intense hybridization in the track containing pituitary derived RNA, at the expected size of PGH mRNA (Figure 4-15). There was no hybridization to the RNA in the remaining tracks visible even after deliberate over-exposure. These results strongly suggest that pig #180 did not express the integrated sequences, and that pig #295 was the only transgenic pig which expressed the foreign gene (4.4.3).

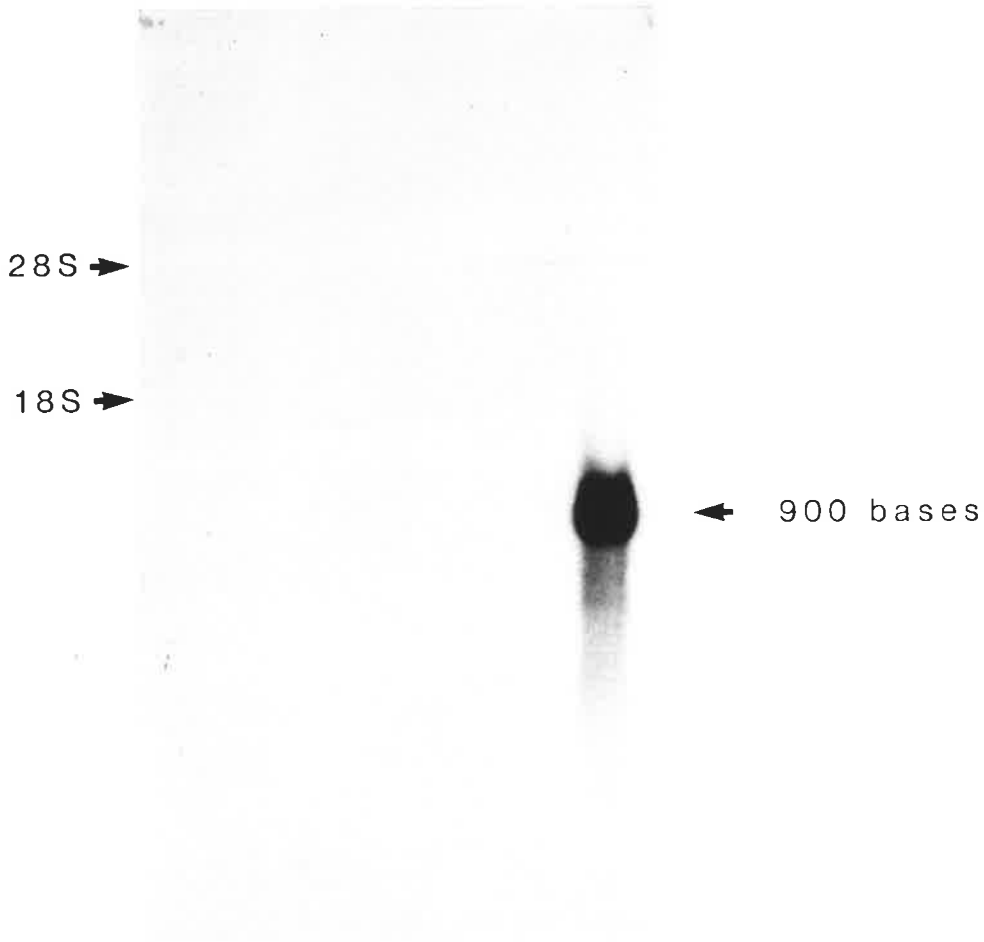
4.5 hMT-IIA promoter manipulation

Previous experiments with both transgenic mice (4.3.3) and pigs (4.4.3) have indicated that the hMT-IIA promoter is active in both of these animals to a sufficient level to induce growth, even in the absence of heavy metals. Similar high "basal" levels of expression have been observed for this promoter in a wide range of tissue culture cell lines (Haslinger and Karin, 1985). As the hMT-IIA promoter has been characterized in great detail by deletion analysis, it was decided to attempt to modify the promoter in a way which would retain its metal responsiveness, but remove the basal level activity. Such a promoter would be ideal for use in animal production, to avoid problems in the handling, and possibly in the fertility (Table 4-2), of overly large breeding stock.

Figure 4-15

Expression of PGH mRNA in transgenic pig #180

Northern blot (6.3.10) containing 10 ug/track of transgenic pig #180 RNA from a number of tissues hybridized to the nick-translated PGH cDNA plasmid, pPG.3 (4.4.4). Liver RNA from a non-transgenic pig was included on the Northern as a negative control.


Control
Pig 180
liver
liver
kidney
spleen
testis
brain
pituitary
28S
18S
900 bases

4.5.1 *Basal sequence deletion*

Regions of the hMT-IIA promoter involved in transcriptional induction by glucocorticoids and by heavy metals have been localized previously by *in vitro* deletion experiments performed by Karin *et al*. (1984b). These studies also identified a 23 bp region between bases -91 and -67 which was responsible for the high level of uninduced expression from this promoter.

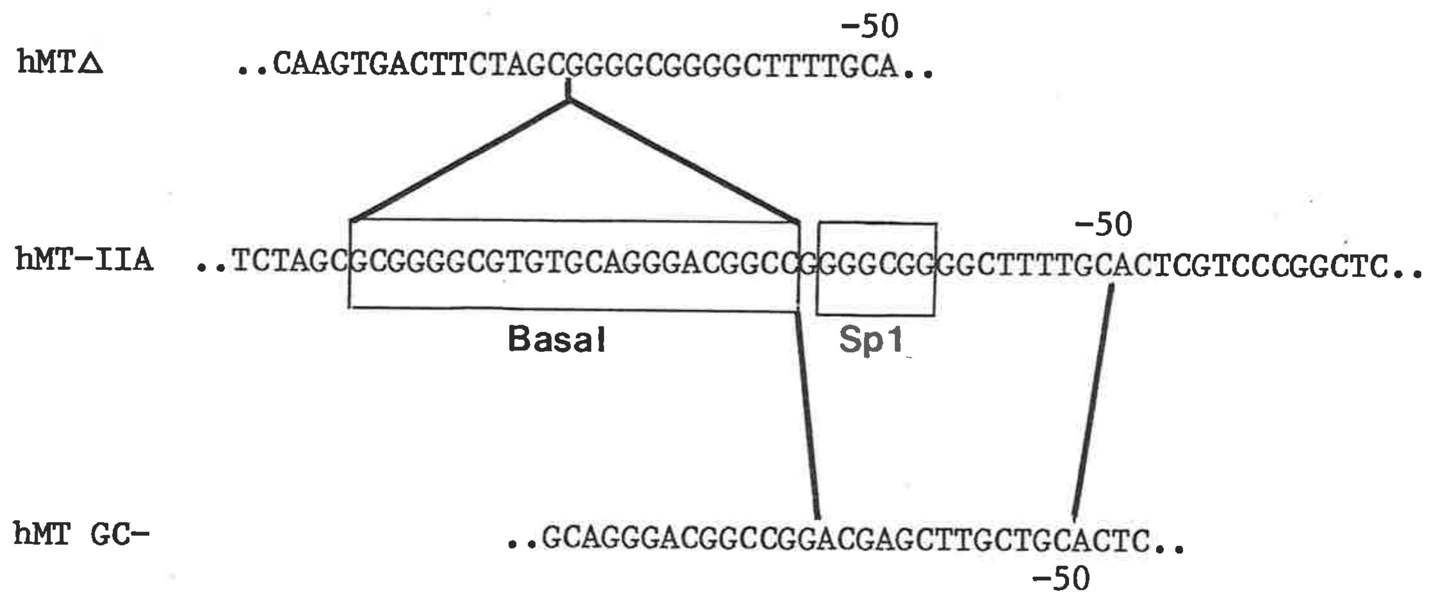
In order to construct a basal-minus hMT-IIA derivative, the 23 bases identified as responsible for this activity were deleted using oligonucleotide directed mutagenesis (6.3.8). The deletion was performed using a 28 base long oligonucleotide, MT.28 (6.2.5), which was complementary to 14 bases either side of the 23 base basal region. Following mutagenesis of the original hMT-IIA/mp8 single-stranded DNA with MT.28, the reaction was transformed into JM101 cells (6.3.6.iii) and the resulting plaques screened for the presence of the deletion by plaque hybridization (6.3.8.iii). Of the 200 plaques screened, 5 % were found to be positive. The accuracy of the deletion was confirmed by nucleotide sequencing. This deleted promoter is referred to as hMTΔ (Figure 4-16).

The hMTΔ promoter was cloned into the pHMPG.4 expression plasmid in place of the normal hMT-IIA promoter by performing a triple ligation (6.3.4.iii) which included the *Hin*dIII/*Eco*RI hMTΔ promoter fragment isolated from RF 'phage, the *Eco*RI insert of pHMPG.4 (Figure 4-3) and *Hin*dIII/*Eco*RI digested pUC19. The promoter and vector fragment were present in equimolar amounts and the *Eco*RI pHMPG.4 insert in a three molar excess. This ligation mixture was transformed into MC1061 cells (6.3.3.v) and plasmid DNA isolated from transformants (6.3.4.ii) examined for the correct restriction pattern. One of the plasmids with the correct restriction pattern was named pHMΔPG.1.

FIGURE 4-16

Nucleotide sequence of altered hMT-IIA promoters

The nucleotide sequence of the two altered hMT-IIA promoters is illustrated alongside the original sequence (4.5.1; 4.5.3). The 23 bp element identified as being responsible for the basal level expression from this promoter (Karin *et al.*, 1984b), and the core of the adjacent Sp1 binding site (Kadonaga *et al.*, 1986) are indicated on the hMT-IIA sequence. The hMTΔ promoter has the 23 bp basal level element deleted (4.5.1), while the hMTGC- construct has the Sp1 binding site replaced with a *Pst*I linker (4.5.3).


-50
hMT△
..CAAGTGACTTCTAGCGGGGCGGGGCTTTTGCA..
-50
hMT-IIA
..TCTAGCGCGGGGCGTGTGCAGGGACGGCCGGGGCGGGGCTTTTGCACTCGTCCCGGCTC..
Basal
Sp1
hMT GC-
..GCAGGGACGGCCGGACGAGCTTGCTGCACTC..
-50

4.5.2 Transgenic mice bearing plasmid pHM△PG.1

i) Production and analysis of transgenic mice

The 2.7 kb HindIII/PvuI insert of pHM△PG.1 was isolated from low melting temperature agarose and used to generate transgenic mice as described (6.3.11). The twenty-eight mice which were produced were examined for the presence of the foreign gene by dot-blot hybridization (6.3.2.iv). 10 ug samples of DNA isolated from tail tissue (6.3.1.ii) from each of the mice was denatured and filtered onto a Gene-screen membrane, which was hybridized to the hMT-IIA promoter HindIII/AvaI probe (4.3.2). This dot-blot indicated that ten of the twenty eight mice were transgenic, containing between one and thirty copies of the pHM△PG.1 insert per cell (Figure 4-17).

ii) Growth of pHM△PG.1 transgenic mice and their offspring

The growth rates of the pHM△PG.1 F0 transgenic mice are illustrated in Figure 4-18. These results demonstrate that most of the transgenic mice actually grew more slowly than their control littermates, a result very different from that obtained with the pHMPG.4 plasmid (Figure 4-7). Of the eleven transgenic mice obtained, only two (A-1 and A-3) are growing faster than their littermates, and only with marginally greater growth ratios. The number of copies of the foreign gene present in the transgenic mice was estimated from plasmid standards present on the original dot-blot (Figure 4-17). These results are illustrated along with the growth ratios of these mice in Table 4-5.

In order to study the zinc induction of growth in pHM△PG.1 transgenic mice, the F0 animals were bred with control animals and the resulting offspring studied for growth induction. The transmission of the pHM△PG.1 gene to offspring was determined by dot-blot analysis of tail DNA samples. A dot-blot illustrating the inheritance of the foreign gene is shown in

FIGURE 4-17

Dot-blot analysis of pHM△PG.1 F0 mice

A membrane containing 5 ug samples of denatured DNA from pHM△PG.1 F0 mice, mouse (MSE) and pig (PIG) negative controls, and pHMPG.4 plasmid positive controls was hybridized to the nick-translated hMT-IIA promoter *Hind*III/ *Ava*I insert probe (4.5.2.i). Ten of the twenty-eight mice tested are transgenic. A key which identifies each of the samples is shown below. The dots in row D contain different amounts of pHMPG.4 plasmid DNA.

	1	2	3	4	5	6	7	8	9	10
A	A-1	A-2	A-3	A-4	A-5	A-6	B-1	B-2	B-3	B-4
B	B-5	B-6	B-7	B-8	C-1	C-2	C-3	C-4	C-5	C-6
C	C-7	C-8	D-1	D-2	D-3	E-1	E-2	E-3	MSE	PIG
D	5pg	10pg	15pg	20pg	25pg	50pg				

1 2 3 4 5 6 7 8 9 10

A

B

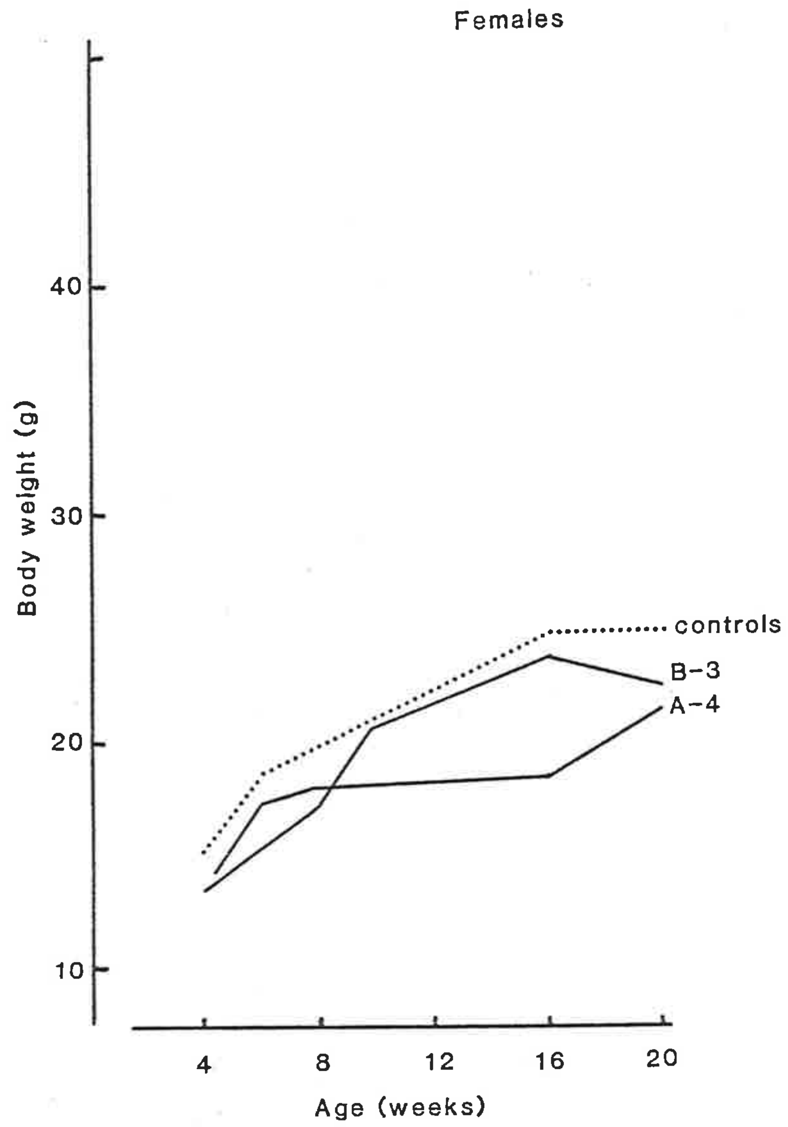
C

D

FIGURE 4-18

Growth rate of pHMΔPG.1 F0 transgenic mice

The growth rate of each of the pHMΔPG.1 transgenic mice, represented as solid lines, is plotted along with the average of sex-matched non-transgenic controls, which are represented as dotted lines (4.5.2).


Females
Body weight (g)
40
30
20
10
controls
B-3
A-4
4
8
12
16
20
Age (weeks)

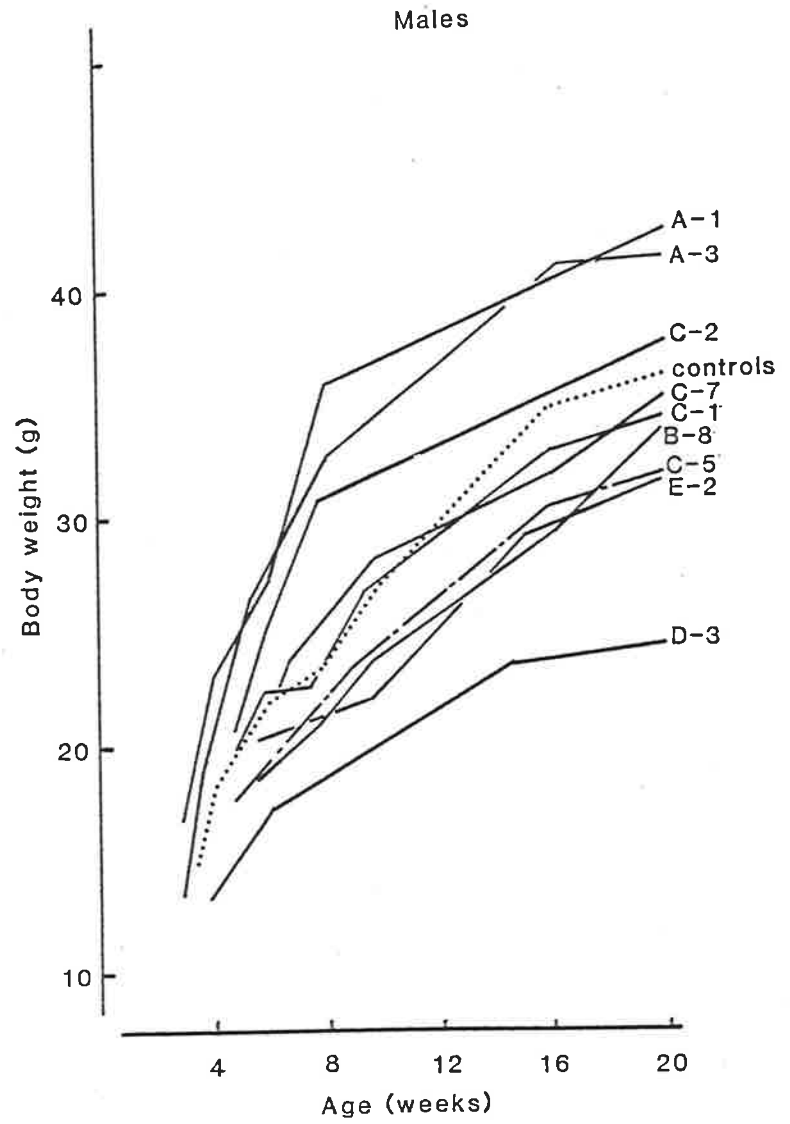

Males
Body weight (g)
40
30
20
10
A-1
A-3
C-2
controls
C-7
C-1
B-8
C-5
E-2
D-3
4
8
12
16
20
Age (weeks)

TABLE 4-5

pHM∆PG.1 F0 mice

The sex, number of copies of integrated foreign genes per cell, and growth ratio at 20 weeks of age of the pHM∆PG.1 F0 mice are illustrated (4.5.2.ii). The gene copy numbers were estimated from the plasmid standards present on the dot-blot illustrated in Figure 4-17, and from a number of other samples which were present on this dot-blot (data not shown).

Animal no.	sex	copy no.	growth ratio
A-1	M	12	1.18
A-3	M	8	1.15
A-4	F	2	0.84
B-3	F	8	0.90
B-8	M	2	0.91
C-1	M	3	0.95
C-2	M	1	1.04
C-5	M	40	0.83
C-7	M	10	0.98
D-3	M	4	0.68
E-2	M	5	0.89

Figure 4-19, and this data is presented in pedigree form in Figure 4-20. As the pedigree indicates, most of the transgenic mice are transmitting their hMT△ /PGH fusion gene to approximately 50 % of their offspring (with the exception of mice B-8 and D-3). The dot-blot (Figure 4-19) demonstrates that DNA from each of the offspring hybridize to the hMT promoter probe to a similar extent as their transgenic parent (with the exception of C-7 offspring). Taken together these results indicate that a lower proportion of the pHM△PG.1 F0 mice are mosaics than was observed for the pHMPG.4 F0 mice.

The pHM△PG.1 transgenic offspring of each parent were divided into two groups, one of which acted as the control (normal water) and the other which was tested for the zinc induction of growth (water supplemented with 25 mM $ZnSO_4$). The growth ratios of these animals was determined at 20 weeks of age and are illustrated in Table 4-6. A surprising result from these experiments was the enhanced growth of many of the transgenic mice, considering the low growth rate of their parent. In each case, with the exception of #B-3, the transgenic mice have sired offspring which grow at considerably greater rates than their own. A particularly clear example of this is the family of mouse #C-5, which produced offspring with growth ratios of up to 1.49, much greater than its own (0.83). A second surprising result from these experiments was the lack of increased growth in animals on a zinc supplemented diet. Although some of the mice show an average increase in size when kept on zinc, there are a number of examples of exactly the opposite result (Table 4-6).

4.5.3 Spl binding site minus hMT-IIA/PGH plasmid, pHMGPG.3

While the work on pHM△PG.1 was in progress information became available indicating that the promoter constructed in Section 4.5.2 would not function as was hoped, but that a different alteration, the destruction of

FIGURE 4-19

Inheritance of foreign pHM△PG.1 genes

The inheritance of the pHM△PG.1 gene introduced into transgenic mice was studied by mating a number of the F0 animals and examining the offspring for the presence of the foreign gene (4.5.2). Dot-blots containing 5 ug samples of both parent and offspring DNA were hybridized to nick-translated hMT-IIA promoter HindIII/AvaI insert DNA. A key describing the origin of each of the samples on this blot is shown below.

a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	*A-3	-3-1	-3-2	-3-3	-3-4	-3-5	-3-6					
B	*B-3	-3-1	-3-2									
C	*B-8	-8-1	-8-2	-8-3	-8-4	-8-5	-8-6	-8-7	-8-8	-8-9	-8-10	-8-11
D	B-8-12	-8-13	-8-14									
E	*C-1	-1-1	-1-2	-1-3	-1-4	-1-5	-1-6	-1-7	-1-8	-1-9	-1-10	-1-11
F	C-1-12	-1-13	-1-14	-1-15	-1-16	-1-17						
G	*C-5	-5-1	-5-2	-5-3	-5-4	-5-5						
H						HUM	MSE	PIG				

b)

	1	2	3	4	5	6	7	8	9	10	11	12
A	*C-7	-7-1	-7-2	-7-3	-7-4	-7-5	-7-6	-7-7	-7-8	-7-9	-7-10	
B	*D-3	-3-1	-3-2	-3-3	-3-4	-3-5	-3-6	-3-7	-3-8	-3-9	-3-10	-3-11
C	D-3-12	-3-13	-3-14	-3-15	-3-16							
D	*E-2	-2-1	-2-2	-2-3	-2-4	-2-5	-2-6	-2-7	-2-8	-2-9	-2-10	-2-11
E	E-2-12	-2-13	-2-14	-2-15	-2-16					PIG	MSE	HUM

A

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

A
B
C
D
E
F
G
H

B

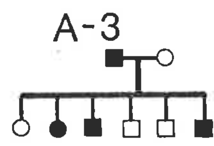
1 2 3 4 5 6 7 8 9 10 11 12

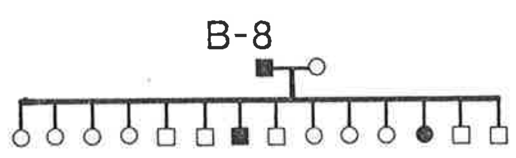
A
B
C
D
E

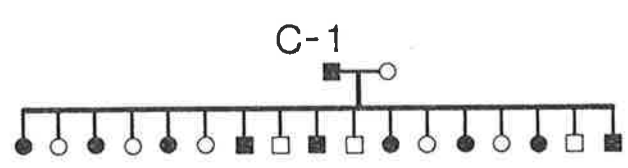
FIGURE 4-20

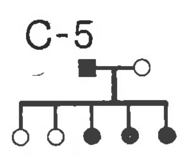
Inheritance of the pHMΔPG.1 foreign genes in transgenic mice

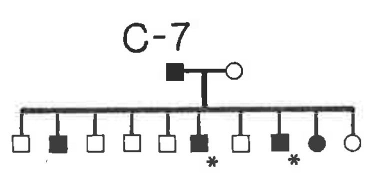
The inheritance data in the dot-blots presented in Figure 4-19 is shown here in pedigree form (4.5.2.ii). Circles represent females and squares males. Solid or open colouring depicts transgenic or non-transgenic animals respectively. Offspring of mouse #C-7 which contain a different number of copies of the foreign gene relative to their transgenic parent (4.5.2.ii) are marked with an asterisk.


A-3


B-8


C-1


C-5


C-7
*
*

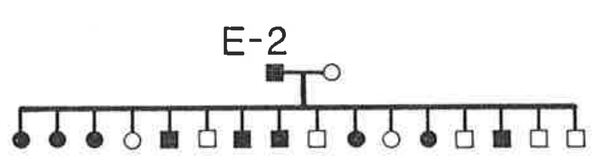

E-2

TABLE 4-6

The effect of zinc on the growth of F1 pHMΔPG.1 transgenic mice

The mean growth ratios of pHMΔPG.1 transgenic mice at twenty weeks of age, both on normal and zinc supplemented diets, are compared to those of their transgenic parent (4.5.2.ii). Diets were supplemented with zinc by dissolving $ZnSO_4$ in the drinking water of these animals to a final concentration of 25 mM. This data does not indicate that there was any consistent growth response to the zinc containing diet. However, many of the F1 animals grew to a much greater size than their transgenic parent.

Founder	Founder Growth ratio	F1 Males		F1 Females	
		+Zn	-Zn	+Zn	-Zn
A-3	1.18	-	1.59	-	1.60
B-3	0.91	-	1.06	-	1.08
C-1	0.95	1.03	1.42	0.93	1.26
C-5	0.83	-	-	1.49	1.28
C-7	0.98	0.83	1.10	-	1.17
E-2	0.89	1.35	1.16	1.45	1.41

a consensus Spl binding site sequence, GGGCGG (Kadonaga et al., 1986) located adjacent to the basal level sequence (Figure 4-16), would generate a promoter with the desired parameters (M.Karin pers. comm.). The nucleotide sequence of an altered hMT-IIA promoter which has the Spl binding site replaced with a PstI linker, and possesses the required transcriptional characteristics, was kindly made available by M.Karin, and is illustrated in Figure 4-16. This alteration was recreated in the hMT-IIA/ mp8 clone by oligonucleotide directed mutagenesis.

A 46 base long oligonucleotide, MT.46 was designed to replace a 15 bp region, surrounding and including the Spl binding site, with a 17 base long PstI linker sequence (Figure 4-16). Following mutagenesis of hMT-IIA/ mp8 single-stranded DNA with MT.46 and transformation into JM101 (6.3.8), plaques containing the correct sequence substitution were selected by plaque hybridization and nucleotide sequencing. RF DNA was isolated from one of the plaques containing the correct mutation and the HindIII/EcoRI insert purified and used in a triple ligation with the EcoRI insert of pHMPG.4 and HindIII/EcoRI digested pUC19 DNA as described above (4.5.2). One of the resulting plasmids from this ligation contained the correct restriction pattern, and was named pHMGPG.3 (GC sequence minus).

The insert of this plasmid is currently being introduced into transgenic mice.

4.6 Discussion

4.6.1 pHMPG.4 Transgenic mice

A 2.7 kb linear HindIII/PvuI fragment containing the hMT-IIA promoter joined to a PGH cDNA/cosmid fusion gene (4.2) was introduced into the germ line of transgenic mice by the microinjection of fertilized one cell embryos (4.3.1, 6.3.11.1). Of the mice which developed from such eggs in

foster mothers, 31 % (20/64) were found to have incorporated the foreign gene (4.3.2.i). The number of copies of the plasmid insert integrated into these mice was determined by slot-blot (4.3.2.ii) analysis and ranged from 1 to 150 copies per cell, which is consistent with the results of others (1.4, 4.1).

The majority of transgenic mice (72 %), 5/8 males and 8/10 females, grew significantly larger than their non-transgenic control littermates (4.3.3). The enhanced growth appeared to be greater in the females (maximum growth ratio 1.91), due to the smaller size of the sex matched controls. As has been previously observed by others, the number of copies of the foreign gene does not appear to be directly reflected in the growth rate of transgenic animals (Palmiter _et al._, 1983; Hammer _et al._, 1984, 1985b; Morrelo _et al._, 1986). For example the two largest females which have identical growth ratios (1.91) have gene copy numbers of 1 (mouse #20) and 150 (mouse #69) copies of the fusion gene per cell (Table 4-1).

The inheritance of the foreign gene was studied by mating the F0 transgenic animals with control mice (4.3.4; Table 4-2). Each of the male mice, with one exception, sired at least one litter of offspring, independent of their growth ratio. Each of the four females with growth ratios of less than 1.2 produced offspring. Of the six females with growth ratios of greater than 1.2, only one (mouse #69) produced any offspring, and none of the three offspring produced by this mouse were transgenic. The infertility of the giant females, and the fertility of the giant males is consistent with the results of Hammer _et al._ (1984), but differ from those of Morello _et al._ (1986). These latter authors have shown that their large mice (expressing HGH) of both sexes can produce offspring by _in vitro_ fertilization rescue and concluded that the inability to sire offspring may be related to some aspect of sexual behaviour rather than a defect in gamete production.

This may explain the ability of female #69 to produce a litter but does not indicate why the litter was so small (three pups). In addition, these pups had to be reared by a foster mother, due to their transgenic mothers inability to produce milk. This result indicates that although they may be able to produce viable gametes, there may be some overriding hormonal defect responsible for the reduced fertility of giant females.

Nine of the twelve mice tested (75 %) passed the gene on to their offspring (4.3.4). The percentage of the offspring of these nine animals which inherited the gene ranged from 7 to 55 % (Figure 4-9). Many of the mice passed the gene on to less than 30 % of their offspring, indicating that they may be germ-line mosaics (Wagner et al., 1983; Palmiter et al., 1984; Soriano and Jaenisch, 1986). In addition to the low frequency of transmission in some instances, a number of the F1 mice contain different copy numbers of the foreign gene to their transgenic parent (4.3.4). This variability in the gene copy number occurs in the families of mice #4, 5, 40, 45, 65 and 67. In three instances (#4, 40 and 45) this variation results in some of the offspring having a lower gene copy number than the parent, while in other cases (#5, 65 and 67) it results in some mice having a higher gene copy number than their parent. This result may also be indicative of these mice being germ line mosaics. The fact that more than one half of the animals tested may be mosaics is surprising, and is a much higher frequency than expected from the literature (1.4, 4.1). The results to date indicate that the F1 pHMPG.4 transgenic mice pass on their foreign genes to one half of their F2 progeny in a normal Mendelian fashion (Figure 4-9), indicating that mosaicism, rather than gene instability is responsible for much of the variability of gene copy number seen in the F1 mice (Figure 4-8).

Many of the F1 mice sired by transgenic parents which had enhanced

growth rates also grew much larger than normal (4.3.4). However the growth ratio of the offspring did not always directly reflect that of their transgenic parent (Table 4-3). For instance mouse #67 (male) had a growth ratio of 0.89, while his male offspring have an average growth ratio of 1.66 (equivalent to the largest of the F0 transgenic males) and female offspring an average growth ratio of 1.58. In two families (#4 and #45) some of the transgenic offspring grew at substantially lower rates than their transgenic parent. Both of these F0 mice are either mosaics, or possess multiple integration sites, as their transgenic offspring differ in the number of copies of the foreign gene they possess. This variation in copy number is linked to some extent with the different growth rates observed within these families. For instance, each of the offspring of mouse #4 which are growing with a ratio of 1.2 or greater possess a high number of copies of the pHMPG.4 gene. Although each of the offspring in this family with a low foreign gene copy number have low growth rates, not all of the offspring with a high copy number show increased growth (Table 4-3). This result may be due to the founder mouse containing the foreign gene integrated into at least two different locations, only one of which, the high copy number locus, is capable of conferring the large growth phenotype. A similar pattern of inheritance is also observed in the offspring of mouse #45, except that in this instance, the offspring with a low foreign gene copy number inherit the large growth phenotype (Table 4-3).

The variation in growth ratios amongst offspring is not confined to animals with gene copy numbers that differ from their transgenic parents (4.3.4; Table 4-3). An example of a mouse which shows no indication of being mosaic but which produced offspring that differ markedly in size, both to their transgenic parent and their own transgenic littermates is mouse #54. The offspring of this mouse (growth ratio 1.25), which all have

an equal number of copies of the foreign gene as their parent, have growth ratios which range from 1.18 to 2.35 (Table 4-3). The hMT-IIA promoter therefore appears to be more active in many of the F1 transgenic mice than in their F0 parent. Variability in the inheritance of the expression of foreign genes linked to the mouse MT-I promoter has been described (Palmiter et al., 1982a). In this instance the altered expression of MT-I/ tk fusion genes in F1 transgenic animals was found to be correlated to some extent with alterations in the methylation pattern of the integrated genes of some animals (Palmiter et al., 1982a). Similar alterations may be responsible for, or associated with the variation observed in the growth of F1 pHMPG.4 transgenic mice.

The results of the transgenic mouse experiments indicate that the pHMPG.4 construct is efficiently integrated into a high percentage (31 %) of the founder mice (4.3.2). Most of the transgenic mice (72 %) express sufficient levels of PGH to result in a substantial increase in growth rate (Table 4-1). Although a large proportion (more than one half) of the F0 transgenic mice may be germ line mosaics (4.3.4), the construct appears to be stable (for at least three generations) and can be transmitted to at least a proportion of the offspring, which in many cases results in increased growth, often much greater than that of the transgenic parent (Table 4-3). The F1 transgenic mice pass on the foreign genes to their F2 offspring in a stable Mendelian fashion (Figure 4-9).

4.6.2 Transgenic pigs

Following the testing of the pHMPG.4 fusion gene (4.2) in transgenic mice (4.3), the 2.7 kb insert of this plasmid was used to generate transgenic pigs (4.4, 6.3.11.ii). A total of seventeen piglets developed from injected eggs, and dot-blot analysis of these revealed that six were transgenic (4.4.2.i). Slot-blot analysis indicated that of these pigs two (pigs

#375 and #739) contained only one half of a copy of the pHMPG.4 insert per cell, while the remaining four pigs (#177, 180, 295 and 736) contained between three (#177) and fifteen (#295) copies of the foreign gene per cell (4.4.2.ii; Figure 4-11).

Southern analysis of BamHI digested DNA from these pigs indicated that each of the animals, with the exception of pig #375, contained all of the copies of the foreign gene integrated into a single chromosomal site (4.4.2.iii). Pig #375 appears to possess three different integration sites, each of which contain much less than one copy of the pHMPG.4 insert. The analysis of EcoRI digests of the transgenic pigs (Figure 4-13) indicated that in one case (pig #177) the copies of the fusion gene had integrated in a random pattern, in another case (#295) in an ordered head to tail configuration, and in a further two instances (#180 and #736) in disordered arrays which may also have undergone rearrangement (4.4.2.iii; Figure 4-13). The integration pattern observed in transgenic mice is usually in head to tail multimers (Palmiter and Brinster, 1985). In the single reported instance of the generation of transgenic pigs, Hammer et al. (1985c) found that in both of the animals studied, ordered patterns of integration had occurred. These same authors also reported that transgenic rabbits contained ordered integration patterns, but that a single transgenic sheep which was studied contained rearranged sequences. The analysis of a larger number of transgenic pigs will be required to determine the frequency of obtaining ordered versus disordered integration of foreign genes in transgenic farm animals. Transgenic mice containing multiple copies of the pHMPG.4 insert all show the normal head to tail integration pattern (McInnes et al., 1987b), indicating that the rearrangement in transgenic pigs is not a function of this particular fusion gene. It is interesting to note that the only transgenic pig which demonstrated an increased growth rate and had an

elevated serum PGH level (4.4.3), was also the only animal containing an ordered head to tail integration pattern (pig #295).

The growth rate of one of the transgenic pigs, pig #295, was much greater than that of controls. This animal grew at 1.18 times the rate of her sex matched littermates (Figure 4-14). This increased growth is clearly demonstrated by the daily weight gain of this animal (from day 50 to 120), 953 grams per day, versus that of her sex matched littermates, with an average of 806 grams per day (Table 4-4). The difference in daily weight gain is even greater if this value is calculated for each of the pigs over their rapid growth phase, when animals increase in weight from 20 to 90 kg. Over this period pig #295 grew at 1273 g/day versus her control littermates which grew at 802 g/day, an increase of over 60 %. This animal reached market weight in only 17 weeks, as opposed to the normal 25 weeks.

None of the other transgenic pigs displayed an enhanced growth phenotype. Two of the transgenic males grew significantly larger than their two very small male control littermates, but were both only slightly larger than the non-transgenic female controls from the other litters. One of the transgenic males, #180, which had a slightly higher concentration of circulating PGH than other animals (4.4.3), was tested for the expression of the foreign gene by Northern analysis (4.4.4). A fusion gene specific oligonucleotide hybridization probe failed to detect any transcripts from the hMT-IIA/ PGH fusion gene in total RNA isolated from liver, kidney, spleen, testis, brain or pituitary tissues (4.4.4). As there was no positive control in this analysis, the Northern was reprobed with a PGH cDNA. This probe clearly detected PGH mRNA in pituitary tissue, but failed to detect PGH mRNA in other tissues (Figure 4-15), even after the deliberate overexposure of the Northern. This result indicates that this animal is not expressing the foreign gene, and that female #295 appears to be the only

transgenic pig containing transcriptionally active hMT-IIA/PGH fusion genes. The lack of expression in other transgenic pigs may be due to the rearrangement of the integrated pHMPG.4 sequences (4.4.2.iii).

4.6.3 hMT-IIA promoter manipulation

The level of uninduced expression from the hMT-IIA promoter was sufficient to promote growth in most transgenic mice, and in one transgenic pig. The optimal promoter for the production of transgenic farm animals would be inducible, with a low level of basal expression. This would solve many problems in animal handling, and if pigs react to the over expression of GH in the same manner as mice (4.3.4), overcome the reduced fertility of females expressing high levels of GH. With this in mind, the modification of the hMT-IIA promoter was attempted (4.5).

The first alteration made was the deletion of the 23 bp element defined as being responsible for the high basal level of transcription from the hMT-IIA promoter by Karin et al. (1984b). This deletion was accomplished by oligonucleotide directed mutagenesis and the deleted promoter inserted into pHMPG.4 in place of the normal hMT-IIA promoter to create plasmid pHM∆PG.1. The 2.7 kb HindIII/PvuI insert of this plasmid was then used to generate a number of transgenic mice.

In contrast to most of the F0 transgenic mice containing the pHMPG.4 insert, most of the F0 pHM∆PG.1 mice were actually smaller than normal size, indicating that the promoter deletion had some effect on the expression of the fusion gene (4.5.2). However, when the F1 offspring of these mice were tested for zinc inducible growth, the surprising result was that 1) F1 pHM∆PG.1 transgenic mice did not display a consistent growth response to zinc, and 2) F1 pHM∆PG.1 transgenic mice often grew at vastly increased growth rates without heavy metal supplemented diets, independent of the growth rate of their transgenic parent (4.5.2.ii), a result similar to that

observed in pHMPG.4 transgenic mice. In a number of instances, F1 pHMΔPG.1 transgenic littermates often grew slower when placed on a zinc containing diet (Table 4-6). The enhanced growth of pHMΔPG.1 F1 transgenic animals on normal diets clearly demonstrates that the 23 bp deletion performed did not remove the sequences responsible for basal level expression. The reason for the slow growth of the pHMΔPG.1 F0 animals is not known.

Information recently became available that the removal of the Sp1 binding site (Kadonaga et al., 1986) of the hMT-IIA promoter would result in the generation of a promoter with a low basal level activity, which was still inducible by heavy metals (M.Karin, pers. comm.). The sequence of a linker scanning mutant which had been generated by M.Karin and shown to have the desired transcriptional properties was therefore recreated using oligonucleotide directed mutagenesis. This altered promoter was inserted into pHMPG.4 in place of the normal promoter, and is currently being used for generating transgenic mice. Once these are generated, and used to produce a number of offspring, testing will begin on the zinc inducibility of this sequence.

CHAPTER 5

FINAL DISCUSSION

Final Discussion

5.1 Expression of PGH in *E.coli*

The effectiveness of growth hormone therapy in enhancing the growth performance of livestock has been known for many years (1.3.2). However, until the advent of recombinant DNA technology insufficient amounts of purified hormone were available for large scale use in the animal industry. The growth hormones of a number of animals have been expressed and purified from the bacterium *E.coli* (1.3.2). Hormone produced in this manner has been shown to be equipotent in promoting growth as pituitary derived hormone (Olson *et al.*, 1981; 1.3.2). In order to create a supply of PGH for use as an anabolic agent in the animal industry, the PGH cDNA clone pPG.3 (the isolation and characterization of which are described in Chapter 2) was cloned into a bacterial expression vector and experiments undertaken to achieve the high level expression of this sequence in *E.coli*.

Two different approaches were undertaken to produce PGH. The first was to express PGH as a pre-hormone, in the hope that the PGH signal sequence would function in *E.coli* cells and direct the secretion and processing of PGH. This was done as the human GH signal sequence was known to function in this manner in *Pseudomonas aeruginosa* and direct the secretion of mature HGH into the periplasmic space (Gray *et al.*, 1984). The second approach was to express methionyl-PGH (m-PGH) in an intracellular fashion, with the PGH signal sequence being replaced with a single amino-terminal methionine.

The construction of a pre-PGH expression vector was achieved by inserting the PGH cDNA sequence into a bacterial expression vector, pKT52, which contained a powerful, regulatable promoter and a bacterial RBS (3.1), to create plasmid pKTGH (Figure 3-1). When this plasmid was transformed into *E.coli* cells no additional protein corresponding to either pre-PGH or mature PGH was seen. As nothing was known at this time about the behaviour

of eukaryotic signal sequences in *E.coli*, this approach was not pursued. Subsequently, a number of reports appeared which described the efficient production and secretion of eukaryotic proteins in *E.coli* by the fusion of eukaryotic coding sequences to prokaryotic signal sequences. A number of different *E.coli* signal sequences have been shown to be functional in this manner, including alkaline phosphatase (Gray *et al.*, 1985; Marks *et al.*, 1986; Dodt *et al.*, 1986), outer membrane protein F (Nagahari *et al.*, 1985) and outer membrane protein A (Hsiung *et al.*, 1986).

The second approach to produce PGH in *E.coli* was intracellular expression. The 75 bases encoding amino acids 2 to 26 of the PGH signal sequence were deleted by oligonucleotide directed mutagenesis, thus joining the first amino acid of the mature PGH molecule directly to the initiator methionine (3.2.2.i; Figures 3-4 and 3-5). This plasmid, pGHX.1, was transformed into *E.coli* cells but failed to produce detectable levels of m-PGH (Figure 3-8). Analysis of RNA from cells containing pGHX.1 revealed that PGH mRNA was being efficiently produced, and indicated that a low level of translation was responsible for the absence of detectable PGH expression (3.2.2.i, 3.2.3). Four different approaches were then undertaken, each of which involved the use of oligonucleotide directed mutagenesis, in an attempt to increase expression levels. They were 1) to express PGH using the two cistron expression system of Schoner *et al.* (1984; 3.2.2.ii), 2) to express PGH as a fusion protein, the non-GH portion of which could be removed by chemical cleavage (3.2.2.ii), 3) to alter the RBS/spacer region of the expression plasmid so as to create a consensus RBS and a "structureless" mRNA spacer (3.2.2.iii), and 4) to alter codons present in the 5' end of the gene which are used infrequently in *E.coli* to those which are used in efficiently expressed *E.coli* genes (3.2.2.iv). The only one of these four approaches which was successful was the alteration of RBS/spacer

sequences. This alteration involved the replacement of the region between the RBS and the initiator ATG in plasmid pGHX.1

i.e. AGGAAACAGACC ATG was replaced with

AGGAGGTAATAT ATG (pGHXS.4) or

AGGAGGTAAAAT ATG (pGHXS.9, see Figure 3-5).

Both of these alterations increased m-PGH expression levels from less than 0.1 % of total protein in cells containing pGHX.1 to approximately 15 % of total cellular protein in cells containing either pGHXS.4 or pGHXS.9 (Figure 3-6).

These plasmids are currently being used to produce sufficient amounts of purified m-PGH to begin field trials on the growth promoting ability of this hormone. In addition to achieving the initial aim of these experiments, i.e. the efficient production of PGH, the experiments described in Chapter 3 highlight the vital role spacer sequences play in the translation of bacterial mRNA. These results may be generally applicable to the expression of eukaryotic genes in *E.coli*, the expression of which is often hindered by inefficient translation (Vize and Wells, 1987).

5.2 Expression of PGH in transgenic animals

The microinjection of cloned DNA into fertilized mouse eggs has been shown to enable the integration and expression of foreign genes in mice (1.4.1, 1.4.2, 4.1). The expression of growth promoting hormones, such as GH or GHRF (1.2), under the control of heterologous promoters has been demonstrated to result in the enhanced growth of transgenic mice (1.4.2). This approach, the introduction of a foreign gene encoding a growth promoting hormone, was undertaken here with the aim of producing transgenic farm animals with enhanced growth characteristics.

The plasmid pHMPG.4 contains a fusion gene consisting of the hMT-IIA

promoter linked to a hybrid gene containing both cDNA and cosmid sequences encoding PGH (4.2). The insert of pHMPG.4 was isolated and used to generate transgenic animals. The first experiments performed utilized mice as a model system to determine the frequency of integration and expression of the pHMPG.4 sequences. Later experiments used the same construct to generate transgenic pigs.

5.2.1 Transgenic mice

A number of mice which developed from eggs injected with the insert of plasmid pHMPG.4 were found to contain between 1 and 150 copies of the construct per cell by dot- and slot-blot analysis (4.3.2). Many of these transgenic mice grew at much greater rates than their non-transgenic littermates, but the growth rates of different transgenic animals were variable. There was no correlation between growth rate and the number of copies of the foreign gene which the animals contained (4.3.3). This result is consistent with the results of others who have observed similar copy number independent growth rates in transgenic mice expressing rat (Palmiter et al., 1982b), human (Palmiter et al., 1983; Morello et al., 1986) or bovine (Hammer et al., 1985a) GH or human GHRF (Hammer et al., 1985b).

These transgenic mice were tested for their ability to transmit the foreign gene to their offspring by crossing a number of these animals with control mice (4.3.4). Only 75 % of the mice tested were capable of passing the foreign gene to their offspring, and in many cases this transmission was to less than one half of their offspring. Transgenic mice usually contain all of the copies of the foreign gene sequence integrated into a single chromosomal site in ordered head to tail arrays, and thus should transmit the foreign gene "loci" to 50 % of their offspring (Palmiter and Brinster, 1985). The low percentage of offspring which inherit the gene from the F0 transgenic mice indicates that many of the F0 animals may be

germ line mosaics (4.3.4, 4.6.1). A number of the F1 transgenic mice were found to contain a different number of copies of the foreign gene to their transgenic parent (4.3.4). These observations indicated that a large proportion (more than one half) of the F0 animals may be mosaics. It is possible that the hMT-IIA/PGH gene construct is unstable in the germ line of mice, and this instability is responsible for the variation in transmission frequency and gene copy number in the F1 animals. However, the stable transmission of the integrated sequences in F1 to F2 animals (Figure 4-9) argues against this possibility, and indicates that for some unknown reason mosaics were generated at a frequency much higher than expected from the literature (4.1, 4.3.4, 4.6.1).

The expression of the foreign gene in F1 animals also varied greatly, with different transgenic mice from the same litter possessing markedly different growth ratios (4.3.4). In some instances this variation appeared to be due to either the F0 parent being a mosaic, or having foreign genes integrated into multiple chromosomal sites, but in others seemed to be due to altered expression patterns in different individuals containing the same foreign genes (4.6.1). In general the F1 mice grew faster than their transgenic parents, with the largest mouse having a growth ratio of 2.35, which is equivalent to the largest HGH transgenic mice produced to-date (Palmiter _et al._, 1983; Morello _et al._, 1986). The reason for this variation in expression is not known. The level and pattern of gene expression is normally inherited in a much more stable fashion (e.g. Hammer _et al._, 1985b), although a number of reports have also found similar variability in the inheritance of foreign gene expression in transgenic mice (e.g. Palmiter _et al._, 1982a; Palmiter _et al._, 1984).

In summary, the pHMPG.4 insert integrates into a high proportion of injected eggs (31 %) and is expressed in the majority (72 %) of transgenic

mice. Although there is some variation in the frequency of transmission of the integrated sequences to transgenic offspring, most (75 %) F0 animals can pass on the gene to at least a proportion of their offspring who then pass on the gene to F2 animals in a stable Mendelian fashion. Most of the mice which inherit the foreign gene also inherit the large growth phenotype. Further breeding studies are required to define the inheritance of expression levels in these animals.

5.2.2 Transgenic pigs

The pHMPG.4 insert was successfully used to generate six transgenic pigs (4.4.1). Two of these animals contained less than 0.5 copies of the foreign gene per cell, and both grew very slowly (4.4.2; Figure 4-14), and will not be discussed further here. The remaining four pigs, two females (#177 and 295) and two males (#180 and 736), were found to contain between 3 and 15 copies of the foreign gene per cell (Figure 4-11; Table 4-4). Of the two females, one (#295) was found to be growing at a substantially increased rate relative to her non-transgenic control littermates (growth ratio 1.18; Figure 4-14; Table 4-4). This pig had a serum PGH level over twice that of other transgenic and control female pigs (Table 4-4). The second transgenic female (#177) was no larger than controls (Figure 4-14). Both of the transgenic male pigs grew faster than their two male control littermates, but both of the control animals were much smaller than normal (4.6.2). When the growth rates of these transgenic males was compared to the female control littermates, only pig #180 was substantially larger (Figure 4-14). However, as this pig does not contain an elevated serum PGH concentration (Table 4-4), or express detectable levels of PGH mRNA in a range of tissues (4.4.4), this increased size is probably sex related rather than due to the expression of the foreign gene. Female #295 therefore appears to be the only transgenic pig expressing the foreign gene

sequence. This animal is also the only transgenic pig which contains all of the copies of the hMT-IIA/PGH fusion gene integrated in head to tail arrays (4.4.2.iii). Both pigs #180 and #736 appear to contain rearranged hMT-IIA/PGH gene sequences, and this may be responsible for the lack of expression in these animals (4.4.2.iii).

Unfortunately, two of the transgenic pigs died during the course of these experiments. The death of the large female #295 was due to a lung infection, and did not appear to be correlated with the expression of the fusion gene in this animal. The transgenic male #180 contracted severe arthritis, one of the symptoms associated with PGH toxicity (1.3.2). However, as this pig did not appear to be expressing PGH from the introduced gene (see above), the development of its disease is probably not related to the animal being transgenic.

In summary, although this data is only preliminary, it indicates that the hMT-IIA/PGH construct is active in at least one of the transgenic pigs, and that the expression of PGH from introduced genes is capable of stimulating a substantial increase in growth rate in pigs.

5.3 Human metallothionein promoter manipulation

The hMT-IIA promoter appears to transcribe sufficient levels of PGH to promote growth in both transgenic mice and transgenic pigs which carry copies of the pHMPG.4 construct, even when not induced with heavy metals (4.6.1, 4.6.2). As the expression of high levels of GH has been shown to cause infertility in transgenic mice (4.3.4, 4.6.1) this uncontrolled expression may be disadvantageous in breeding stock. Exposure to high levels of PGH has been shown to lead to liver and kidney damage in pigs (1.3.2). As some of the giant pHMPG.4 transgenic mice appear to have liver damage (A.Michalska pers. comm.) the uncontrolled expression of PGH in transgenic

pigs may be deleterious. The handling of overly large breeding stock could also prove to be difficult. It was therefore decided to attempt to modify the hMT-IIA promoter in such a way as to create a promoter which is only transcribed in the presence of an inducer, thus allowing the avoidance of all of the complications discussed above.

The experiments of Karin et al. (1984b) identified a 23 bp sequence responsible for the high level of basal expression from this promoter (4.5). This 23 bp element was deleted from the hMT-IIA promoter by oligonucleotide directed mutagenesis and the altered promoter substituted for the normal promoter present in pHMPG.4. The plasmid containing the altered promoter was named pHM△PG.1 (4.5.1). The insert of this plasmid was then used to generate a number of transgenic mice (4.5.2).

Initial results indicated that the deletion of this 23 bp basal sequence had generated a promoter with the desired characteristics, as only one of the ten F0 transgenic mice generated with this construct grew at an increased rate relative to its non-transgenic littermates (4.5.2.ii; Figure 4-18). However, when a number of these mice were bred to produce offspring for zinc induction studies, a number of the F1 mice were found to grow at substantially increased rates compared to those of their transgenic parent (4.5.2.ii; Table 4-6). In addition, there was no consistent growth response to diets containing increased zinc levels in these mice. These results clearly indicate that the 23 bp deletion did not remove the sequences responsible for the basal level of expression from this promoter. Although the increased expression of the foreign gene in pHM△PG.1 F1 animals is consistent with the results obtained with pHMPG.4 F1 mice (5.2.1), there is no obvious explanation for the lack of expression in the F0 pHM△PG.1 animals.

A second promoter alteration has been made, which replaces the Sp1

binding site of the hMT-IIA promoter with a linker sequence (4.5.3). Such an alteration has been shown to generate a promoter which possesses the desired characteristics in tissue culture systems (M. Karin, pers. comm.). This altered promoter has been inserted into pHMPG.4 in place of the normal promoter to create plasmid pHMGPG.3 (4.5.3), and is currently being used to generate transgenic mice.

5.4 Future work

Future work on the bacterial expression of PGH will focus on the construction of plasmids which secrete PGH into the periplasmic space of *E.coli*. This may be achieved by modifying the pre-PGH expression plasmid pKTGH in a similar fashion to that performed to generate the RBS/spacer altered intracellular expressing plasmids pGHXS.4 and pGHXS.9 (3.2.2.iii). Alternatively, the cDNA sequences encoding mature PGH could be fused to sequences encoding an *E.coli* signal sequence. This form of expression may have a number of advantages over intracellular expression, including ease of purification of the recombinant product as there are very few *E.coli* proteins present in the periplasmic space (Gray *et al.*, 1985; Hsiung *et al.*, 1986).

There are two immediate aims in the transgenic animal experiments. They are to produce a larger number of transgenic pigs with the pHMPG.4 construct, in order to more accurately define the activity and effect of this construct in transgenic pigs, and secondly to test the effectiveness of the pHMGPG.3, Sp1 site altered plasmid, for basal level expression and zinc induction. Although the only index studied in the experiments presented here was growth, the analysis of transcription levels and transcriptional control of the different constructs will be performed and may provide useful information about the regulation of the fusion gene in these

animals. It is possible that the hMT-IIA promoter may prove to be unsuitable for regulated expression, and other promoters may need to be tested.

In addition to utilizing different promoter sequences, constructs expressing different growth promoting hormones may be used. The expression of human GHRF in transgenic mice has been shown to stimulate growth (Hammer et al., 1985b; 1.4.2). A construct which expresses human GHRF would probably also stimulate growth in pigs (1.2.1). Alternatively, as the protein sequence of porcine GHRF is known, and due to the high conservation of this hormone across species, it would require only minor alterations to adapt the human gene to produce the porcine protein (1.2.1). A second alternative would be to express insulin-like growth factor I, which may also effectively promote growth in transgenic animals (1.2.3).

Two of the transgenic pigs (#177 and #180) have been crossed to control animals, and the offspring of these animals will be examined for expression of the integrated gene sequences. It is possible that some of these offspring may express the foreign gene, even though their transgenic parent did not, if the hMT-IIA promoter behaves in the same fashion in transgenic pigs as it does in transgenic mice. This is unlikely in the case of pig #180, which appears to contain rearranged foreign gene sequences (4.4.2).

Experiments aimed at producing other transgenic animals, namely sheep, cattle and goats, are also underway.

The experiments described in this thesis demonstrate for the first time that it is possible to produce transgenic farm animals with improved growth performance. Further modification of gene control sequences may be required before in vivo control of the introduced genes can be achieved. Nevertheless, the results obtained so far are encouraging, and suggest that substantial increases in livestock production may be achieved by the transgenic animal approach.

CHAPTER 6

MATERIALS AND METHODS

6.1 Abbreviations

APS:	ammonium persulphate
BCIG:	5-bromo-4-chloro-3-indolyl- ß -D-galactopyranoside
bisacrylamide:	N,N'-methylene-bis acrylamide
ddNTP:	dideoxynucleoside triphosphate
DNase:	deoxyribonuclease
DTT:	dithiothreitol
IPTG:	isopropyl- ß -D-thio-galactopyranoside
PEG:	polyethylene glycol (MW 6000)
RNase:	ribonuclease
SDS:	sodium dodecyl sulphate
Temed:	N,N,N',N'-tetramethylethylenediamine
TCA:	trichloro-acetic acid
TMACl:	tetramethylammonium chloride

Additional abbreviations are as described in "Instructions to Authors" (1978) Biochem. J. 169, 1-27. All rotor serial numbers correspond to Beckman centrifuge rotors.

6.2 Materials

6.2.1 General reagents and materials

Reagents used were generally of analytical grade, or the highest available purity. Most chemicals were obtained from a number of suppliers, the major source of the more important materials are listed below:

Acrylamide: Sigma Chemical Co.

Agarose (low melting temperature): B.R.L. Inc.

Ampicillin: Sigma Chemical Co.

BCIG: Sigma Chemical Co.

Bisacrylamide: Sigma Chemical Co.

Caesium Chloride: K.B.I.

ddNTPs: Pharmacia

dNTPs and NTPs: Sigma Chemical Co.

Gene-screen: New England Nuclear

Glyoxal: B.D.H. Ltd.

IPTG: Sigma Chemical Co.

Mixed bed resin (AG 501-X8(D)): Bio-rad

Nitrocellulose: Schleicher and Schuell

Nonidet P40: B.D.H. Ltd.

Sephadex G-50 (fine): Pharmacia

TMACl: Tokyo Kasei Kogyo Co. Ltd.

"Trizma base": Sigma Chemical Co.

Zetaprobe: Bio-rad

6.2.2 Enzymes

Enzymes used in the course of this work were obtained from the sources listed below:

AMV reverse transcriptase: Molecular Genetics Inc.

Calf intestine alkaline phosphatase (CIP): Boehringer-Mannheim

DNase I (bovine pancreas): Sigma Chemical Co.

E.coli DNA polymerase I: Biotechnology Research Enterprises, S.A. (BRESA)

E.coli DNA polymerase I, Klenow fragment: BRESA

Exonuclease BAL-31: New England Biolabs

Lysozyme: Sigma Chemical Co.

Proteinase K: Boehringer-Mannheim

Restriction endonucleases: New England Biolabs and Pharmacia

Ribonuclease A: Sigma Chemical Co.

Ribonuclease H: B.R.L.

RNase free DNase: Promega

T4 DNA ligase: BRESA

T4 polynucleotide kinase: Boehringer-Mannheim

6.2.3 Isotopically labelled compounds

α-^{32}P-dNTPs (1800 Ci/mmole): BRESA

γ-^{32}P-ATP (2000 Ci/mmole): BRESA

6.2.4 Bacterial strains and media

i) Strains

E.coli MC1061: *araD139*, Δ(*ara*, *leu*) 7697, Δ*lacX74*, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA* (Casadaban and Cohen, 1980)

E.coli JM101: Δ(*lac-pro*), F' *lacI*^{q} Z ΔM15, *traD1* (Messing, 1979)

ii) Media

L-Broth (Luria broth): 1% (w/v) bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, pH 7.0. When appropriate ampicillin (100 ug/ml), tetracycline (20 ug/ml) or chloramphenicol (170 ug/ml) were added.

L-agar plates: L-broth containing 1.5% (w/v) bacto-agar (Difco)

Minimal medium: 2.1% (w/v) K_2HPO_4, 0.9% (w/v) KH_2PO_4, 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) tri-sodium citrate, 0.4% (w/v) glucose, 0.2% $MgSO_4$ and 0.0001% (w/v) thiamine.

Minimal plates: Minimal medium containing 1.5% bacto-agar.

2 X YT broth: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH7.0.

All media were prepared with distilled water and sterilized by autoclaving.

6.2.5 Oligonucleotides

All oligonucleotides were synthesized by BRESA .The sequence of the oligonucleotides used in this work were as follows:

*Eco*RI linker: 5' dGGAATTCC 3'

GH.23: 5' dGGATGGAGTAACGCTGTCCCTCC 3'

GH.25: 5' dCAGCCAGTTGGTGCAGGTGCTGGGC 3'

GH.27: 5' dGCCATCTTCCAGCTCCCGCATCAGGGC 3'

GH.30: 5' dGGGCATGGCTGGGAACATGGTCTGTTTCCT 3'

GH.34: 5' dCGGCGTTGGCGAACAGGCTGGACAGCGGCATGGC 3'

GH.38: 5' dCATGGCTGGGAACATAT A/T TTACCTCCTGTGTGAAATTG 3'

GH.42: 5' dCAAGGGCATGGCTGGGAAACCGTTATCATCCTCCATGGTCTG 3'

GH.50: 5' dGGGCATGGCTGGGAACATTTAATCATCCTCCATGGTCTGTTTCCTGTGTG 3'

MT.26: 5' dAGGCGGCTAGAGTCGGGACAGGTTGC 3'

MT.28: 5' dCAAAAGCCCCGCCCCGCTAGAAGTCACT 3'

MT.46: 5' dGAGCCGGGACGAGTCCTGCAGCCAAGCTCGTCGGCCGGGCGCTGCC 3'

USP: 5' dGTAAAACGACGGCCAGT 3'

SEQ.1: 5' dGAGGGAGGCCCCTGCTCC 3'

SEQ.2: 5' dGTGAGGGGGACGCCCACC 3'

SEQ.3: 5' dGAACCGCGCCCCAGTGTAG 3'

SEQ.4: 5' dGAGAAGCTGAAGGACCTGGAG 3'

6.3 Methods

6.3.1 Isolation of DNA

i) Isolation of plasmid and cosmid DNA

A single bacterial colony containing the plasmid of interest was picked from an L-agar plate, containing the appropriate antibiotic, into 100 ml of L-broth plus antibiotic and grown overnight at 37 ^{o}C with vigorous shaking. On the following day the cells were harvested by centrifugation

and plasmid isolated by lysis using lysozyme and Triton-X-100 followed by centrifugation through CsCl gradients.

Cells were collected by spinning at 6000 rpm (Beckman, JA-20 rotor) for 5 minutes then resuspended in 4.5 ml of a solution containing 15% (w/v) sucrose, 20 mM Tris-HCl pH8.0, 5 mM EDTA, 1 mg/ml lysozyme and kept on ice for 5 minutes. 0.5 ml of 500 mM EDTA was then added and the cells kept on ice for a further 5 minutes. 5.0 ml of 0.2% (v/v) Triton-X-100 was then added and after thorough mixing incubated on ice for 10 minutes. Chromosomal DNA and cellular debris was removed by centrifugation at 16,000 rpm (JA-21 rotor, 4°C) for 45 minutes. The supernatant was collected and made up into a CsCl/ethidium bromide gradient.

Gradients were formed by adding 8.0 grams of solid CsCl to 8.0 ml of cleared lysate and 150 ul of 10 mg/ml ethidium bromide, followed by centrifugation at 45,000 rpm (Ti-50 rotor, 20°C) for 40 hours. The lower band was collected from each gradient and the ethidium bromide removed by two butan-1-ol extractions. Two volumes of TE buffer (10 mM Tris-HCl , 1 mM EDTA, pH7.5) were then added and the solution phenol/chloroform extracted (1:1). Two volumes of ethanol were then added and the DNA collected by centrifugation (10,000 rpm, JA-20, 4°C, 15 min). Purified DNA was resuspended in TE buffer and stored at 4°C. Approximately 2 ug of plasmid was recovered for each ml of overnight culture used.

ii) <u>Isolation of DNA from animal tail samples</u>

Tail samples were obtained from mice at approximately four weeks of age (40 mg of tissue) and from pigs almost straight after birth (500 mg of tissue) and stored frozen until required. Tail samples were disrupted by grinding in liquid nitrogen then resuspended in a solution containing 1% (w/v) SDS, 50 mM Tris-HCl pH7.5, 10 mM EDTA and 50 ug/ml proteinase K (1.0 ml for mouse tails and 10.0 ml for pig tails), and incubated at 37°C

for 2 to 3 hours. Three phenol/chloroform extractions were then done and the nucleic acids collected by ethanol precipitation. The sample was then resuspended in TE buffer and digested with RNase A (50 ug/ml) for 60 minutes at 37 ^{o}C. After RNA hydrolysis the sample was phenol/chloroform extracted and the DNA collected by ethanol precipitation. The DNA was redissolved in TE buffer (200 ul for mice, 2.0 ml for pigs) and the 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 1 ug of each sample on an agarose mini-gel (6.3.2.ii). Approximately 1 ug of DNA was recovered for each mg of starting material.

6.3.2 Restriction enzyme digestion and analysis of DNA

i) Restriction enzyme digestion

Restriction enzyme digests were performed using the conditions described by Davis *et al.* (1980) or as recommended by the supplier. Analytical digests were done in 5 ul reactions containing 100-200 ng of DNA and a two to four-fold excess of enzyme. Preparative digests were done in 20 ul reactions containing 2-5 ug of DNA and a two to four-fold excess of enzyme.

Reactions were terminated by adding a one third volume of 3 X urea loading buffer (4 M urea, 50% (w/v) sucrose, 50 mM EDTA pH8.0, 0.1% (w/v) bromocresol purple) and the sample loaded onto either an agarose (6.3.2.ii) or polyacrylamide gel (6.3.2.iii). Alternately, a phenol/chloroform extraction was performed by adding an equal volume of phenol/chloroform (1:1) and vortexing, followed by centrifugation (one minute, Eppendorf) and recovery of the aqueous phase. This was then ethanol precipitated by the addition of one tenth volume of 3 M Na-acetate (pH5.4) and two volumes of ethanol, chilling on ice for five minutes, then spinning in an Eppendorf centrifuge

for 10 minutes. Following removal of the ethanol the pellet was washed with 500 ul of 70 % (v/v) ethanol, vacuum dried, and resuspended in TE buffer.

ii) Agarose gel electrophoresis

Analytical

Agarose (0.65-1.5% (w/v)) was dissolved in 1 X TEA buffer (40 mM Tris-acetate, 20 mM Na-acetate, 1 mM EDTA pH8.2) by boiling and cast onto either 75 mm X 50 mm microscope slides or 100 mm X 80 mm glass plates. Samples were loaded in 1 X urea load and electrophoresed submerged in 1 X TEA at 100 mA constant current until the tracking dye neared the bottom of the gel. DNA was visualized by staining the gel with 10 ug/ml ethidium bromide for five minutes and viewing under UV light (254 nM).

Preparative

Low melting temperature agarose was dissolved in 1 X TEA buffer and cast onto 75 mm X 50 mm slides as described above. Samples were loaded in 1 X urea load and run at 50 mA at 4°C. The DNA was then stained with ethidium bromide and the DNA visualized under long wave (304 nM) UV light. The required band was excised with a scalpel, placed in a tube containing 200 ul of TE buffer and heated to 70°C for 10 minutes. One phenol and one phenol/chloroform extraction were then done and the DNA collected by ethanol precipitation, and resuspended in TE buffer. Typical sample recovery was approximately 50%.

iii) Polyacrylamide gel electrophoresis

Electrophoresis of DNA fragments less than 500 bp in length, was carried out in vertical 140 X 140 X 0.5 mm gels containing, usually, 6% (w/v) acrylamide/ bisacrylamide (29:1, w/w), polymerized in 1 X TBE buffer (90 mM Tris-borate, 2.5 mM EDTA pH8.3), by the addition of 0.1% (w/v) APS and 0.1% (v/v) temed. Electrophoresis was performed at 300 volts for approximately 90 minutes after samples had been loaded in 1 X urea load.

Following staining with ethidium bromide the DNA was visualized under short wave UV light (254 nM).

iv) Transfer to, and hybridization of DNA immobilized on membranes

Transfer to nitrocellulose membranes

Plasmid or cosmid DNA that had been digested with restriction endonucleases and fractionated by electrophoresis was transferred to nitrocellulose using the method of Southern (1975) with the addition of a short (e.g. 10 min.) pre-treatment with 0.25 M HCl (Wahl *et al.*, 1979).

Following transfer membranes were air dried then baked at 80 ^{o}C *in vacuo* for 2 hours. After baking, filters were sealed in a plastic bag and prehybridized for 2-16 hours in a solution containing 50% (v/v) formamide, 5 X SSC (1 X SSC is 150 mM NaCl, 15 mM Na_3 citrate), 50 mM potassium phosphate buffer pH6.5, 100 ug/ml sonicated salmon sperm DNA, 5 X Denhardts (1 X Denhardts contains 0.02% (w/v) each of BSA, Ficoll and polyvinyl-pyrrolidone) at 42 ^{o}C. Radioactive probes were heat denatured by boiling for 10 minutes and added to the bag containing the prehybridized filter. Hybridization was carried out for 16-24 hours at 42 ^{o}C.

Following hybridization, filters were washed in 2 X SSC at room temperature for 10 minutes and three times with 0.1 X SSC, 0.1% (w/v) SDS at 65 ^{o}C for 30 minutes each .

Washed filters were sealed in plastic and exposed to X-ray film at -80 ^{o}C in the presence of a tungstate intensifying screen.

Transfer of DNA to Zeta-probe nylon membranes

DNA fragments fractionated by gel electophoresis were transferred to Zeta-probe membranes by the alkali blotting procedure described by Chomczynski and Qasba (1984) as modified by Reed and Mann (1985). Following electrophoresis, gels were treated with 0.25 M HCl for 10 minutes then allowed to transfer overnight using 0.4 M NaOH as the transfer buffer.

After overnight transfer filters were sealed in plastic bags and prehybridized overnight at 42 ^{o}C in a solution containing 0.9 M NaCl, 0.5% (w/v) skim milk powder, 50% (v/v) formamide, 50 mM Tris-HCl pH7.5, 10% (w/v) dextran sulphate, 1% (w/v) SDS and 500 ug/ml sonicated/denatured salmon sperm DNA. Heat denatured probes were then added and hybridization at 42 ^{o}C carried out for 16-24 hours. Filters were washed in 2 X SSC at room temperature for 10 minutes then washed three times with 0.1 X SSC, 0.1% SDS at 65 ^{o}C (60 min. each), wrapped in plastic and autoradiographed.

Dot blots

DNA samples (from 2-5 ug) were added to 200 ul of 2 M NaCl, 0.1 M NaOH and boiled for three minutes. The samples were then chilled on ice and neutralized by adding 25 ul of 3 M Na-acetate pH5.6. The denatured DNA was then filtered onto Gene-screen membranes using a Schleicher and Schuell dot blot apparatus. After washing the sample through with 400 ul of 2 M NaCl the filter was air dried and the DNA fixed by baking at 80 ^{o}C for two hours.

Dot blots were prehybridized at 42 ^{o}C overnight in a solution containing 0.9 M NaCl, 50% (v/v) formamide, 10 X Denhardts, 1% (w/v) SDS, 50 mM Tris-HCl pH7.5, 10% (w/v) dextran sulphate and 100 ug/ml sonicated/ denatured salmon sperm DNA. Heat denatured ^{32}P-labelled probe was then added and hybridized for 16-24 hours at 42 ^{o}C. The filter was then washed as described for Zeta-probe blots.

6.3.3 Construction of a porcine pituitary cDNA library

i) Isolation of RNA from porcine pituitaries

Porcine pituitaries which had been snap frozen in liquid nitrogen directly after removal from slaughtered animals were ground to a fine powder using a mortar and pestle. The ground cells were resuspended in 10 ml of a solution containing 4 M guanidine isothiocyanate, 1% (w/v) sarko-

syl, 0.1 M ß-mercaptoethanol, 50 mM Tris-HCl pH7.5 and passed through a French tissue press to shear high molecular weight DNA. RNA was precipitated by the addition of a one half volume of ethanol followed by freezing and centrifugation at 10,000 rpm (JA-20 ,4 ^{o}C) for 10 minutes. The pellet was then resuspended in the guanidine isothiocyanate solution and re-precipitated. The pellet was then resuspended in 10 ml of a solution containing 7 M urea, 10 mM EDTA, 0.1% SDS and phenol/chloroform extracted twice. The RNA was then ethanol precipitated, washed with 2 M LiCl, washed with 70% (v/v) ethanol, spun down and vacuum dried.

Gel electrophoresis of RNA

The integrity of the purified RNA was checked by denaturing a sample with glyoxal and electrophoresis through an agarose gel run in 10 mM Na-phosphate (pH6.5) buffer (6.3.9.iii). After staining with ethidium bromide intact 18S and 28S rRNA bands were clearly visible.

Isolation of poly A^{+} RNA and Northern analysis

Polyadenylated RNA (poly A^{+}) was isolated from total RNA by two passages over oligo-dT cellulose by the method described by Aviv and Leder (1972). The integrity of poly A^{+} RNA used in the construction of the cDNA library was checked by Northern analysis. Glyoxal treated RNA was fractionated by electrophoresis though an agarose gel (6.3.9.iii), transferred to nitrocellulose (6.3.9.iii), followed by hybridization to the PGH specific oligonucleotide GH.27 (6.2.5) which had been kinased with ^{32}P as described (6.3.8.iii). Autoradiography of this filter detected a single intense band of approximately 800 bases in length (data not shown).

ii) cDNA synthesis

A modified version of the RNase H procedure for cDNA synthesis described by Gubler and Hoffman (1983) was used to convert RNA to double stranded cDNA. For first strand synthesis 5 ug of poly A^{+} RNA was resus-

pended in 7 ul of H_2O. 1 ul of 1 M KCl, 2 ul of 1 M Tris-HCl pH8.3, 2 ul of 100 mM $MgCl_2$, 2 ul of 100 mM DTT, 1 ul each of 20 mM solutions of all four dNTPs (dATP, dTTP, dGTP and dCTP), 0.5 ul of 1 mg/ml oligo dT_{10}, and 1 ul of 20 U (units) /ul AMV reverse transcriptase were then added and the reaction incubated at 42 ^oC for 60 minutes. The reaction was then terminated by phenol/chloroform extraction and the nucleic acids ethanol precipitated.

The RNA/cDNA hybrid was resuspended in 60 ul of H_2O along with 10 uCi of $\alpha - {}^{32}P$-dATP, and the second cDNA strand synthesized by adding 10 ul of 1 M KCl, 10 ul of 100 mM $(NH_4)_2SO_4$, 5 ul of 100 mM $MgCl_2$, 5 ul of 1 mg/ml BSA, 2 ul of 1 M Tris-HCl pH7.5, 1 ul of 10 mM DTT, 1 ul each of 20 mM stocks of all four dNTPs, 3 ul of 10U/ul DNA polymerase I, 1 ul of 1U/ul RNase H and 1 ul of 1U/ul T4 DNA ligase. The 100 ul reaction mix was incubated at 12 ^oC for 60 minutes then at 22 ^oC for 60 minutes. The reaction was terminated by phenol/chloroform extraction and the cDNA precipitated out of 2 M ammonium acetate by adding a one half volume of 4 M ammonium acetate and two volumes of ethanol followed by centrifugation at 10,000 rpm (Eppendorf centrifuge) at 4 ^oC for 10 minutes. After washing with 70% (v/v) ethanol the pellet was vacuum dried and redissolved in TE buffer.

The amount of second strand cDNA synthesized was estimated by determining the acid insoluble counts by TCA precipitation. A sample of the second strand reaction mix was added to a tube containing 20 ul of 1 mg/ml BSA and 1.2 ml of 10% (w/v) TCA, incubated on ice for 10 minutes then filtered through a Whatman GF/A glass fibre filter disc. The number of precipitated counts was determined by Cherenkov counting and the percentage of incorporated counts estimated and used to calculate the amount of second strand synthesized. Yields ranged from 1 to 2 ug of double stranded cDNA from 5 ug of input RNA.

iii) Klenow end-fill and methylation of cDNA

Although the cDNA synthesized in the above step should be blunt-ended it was found that a Klenow end-fill substantially increased the ability of this cDNA to self- ligate. The end-fill reaction was done in a 20 ul reaction volume containing 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 uM of all four dNTPs, 100 ug/ml BSA, 3U of DNA polymerase I Klenow fragment and approximately 500 ng of cDNA at 37 ^{o}C for 30 minutes. Following phenol/ chloroform extraction the blunt ended cDNA was ethanol precipitated.

Internal *EcoRI* restriction sites were blocked by treating the cDNA with *EcoRI* methylase. The 500 ng of end-filled cDNA was methylated in a 20 ul reaction volume which consisted of 2 ul of 1 M Tris-HCl pH8.0, 0.8 ul of 250 mM EDTA, 2 ul of 800 uM S-adenosyl-L-methionine, 2 ul of BSA, 12 ul of cDNA in TE buffer and 1 ul of *EcoRI* methylase (20U/ul) and was incubated at 37 ^{o}C for 30 minutes. The reaction was terminated by phenol/ chloroform extraction then ethanol precipitated, washed with 70% (v/v) ethanol, vacuum dried and resuspended in 20 ul of TE buffer.

iv) Linker ligation

The 8 base synthetic *EcoRI* linkers used (6.2.5) were phosphorylated separately with both γ - ^{32}P-ATP and cold ATP before use in ligations .

Linkers were kinased with ^{32}P by lypholizing 50 uCi of γ- ^{32}P-ATP in a tube and resuspending with 6 ul of H_2O. 1 ul of 10 mM DTT, 1 ul of 10 X K (500 mM Tris-HCl pH7.5, 100 mM $MgCl_2$), 1 ul *EcoRI* linkers (50 ng/ul) and 1 ul of 1U/ul T4 polynucleotide kinase were then added and the reaction incubated at 37 ^{o}C for 60 minutes. The reaction was terminated by heating to 70 ^{o}C for 5 minutes then cooled slowly to room temperature and stored at 4 ^{o}C. Linkers were kinased using cold ATP as described above except that 1 ul of 10 mM ATP was substituted for the γ- ^{32}P-ATP, and 500 ng of linkers and 4 U of polynucleotide kinase were used .

Linkers were ligated onto blunt-ended, methylated cDNA in a 10 ul reaction containing 1 ul cold kinased linkers (50 ng), 1 ul ^{32}P- kinased linkers (5 ng), 5 ul of cDNA (100 ng), 1 ul of 10 X L buffer (100 mM Tris-HCl pH7.5, 100 mM $MgCl_2$, 10 mM DTT), 1 ul of 10 mM ATP and 1 ul of 1U/ul T4 DNA ligase. The ligation was incubated at 4^oC overnight.

On the following day 75 ul of H_2O and 10 ul of 10 X EcoRI buffer were added and the reaction heated to 70^oC for 5 minutes. A 5 ul sample was then removed and stored at -20^oC. 5 ul of 20U/ul EcoRI was added to the remaining 90 ul and incubated at 37^oC for 2 hours. A second 5 ul sample was then removed and the remaining 90 ul stored at -20^oC. The two 5 ul samples were then analyzed on a 10% polyacrylamide gel (6.3.2.iii) to determine the extent of ligation and of recutting.

The remaining 90 ul of the linker-ligation-recutting reaction was phenol/chloroform extracted, ethanol precipitated, then resuspended in 10 ul of TE. 3 ul of urea load was then added and the linker monomers removed by electrophoresis through a low melting temperature agarose gel. Molecular weight markers were run on the same gel and the cDNA of between 400 bp and 1.5 kb in size was excised and purified (6.3.2.ii).

v) Ligation and transformation of cDNA

Preparation of vector and ligation

The plasmid used for library construction was pUC19 (Norrander *et al.*, 1983). Plasmid (5 ug) which had been purified by banding in CsCl (6.3.1.i) was cut to completion with a five-fold excess of EcoRI. The 5' terminal phosphates were then removed by adding Tris-HCl pH8.0 to a final concentration of 100 mM plus 0.1 U of CIP and incubating at 37^oC for 60 minutes. The linear, dephosphorylated vector was electrophoresed through a low melting temperature agarose gel (6.3.2.ii) to remove the phosphatase and any contaminating supercoiled plasmid.

The linkered cDNA was ligated to the dephosphorylated vector in a 10 ul reaction containing 20 ng of vector, 2 ng of cDNA, 1 mM ATP, 1 mM DTT, 10 mM Tris-HCl pH7.5, 10 mM $MgCl_2$ and 0.1U of T4 DNA ligase which was incubated at 14°C for 2 hours.

Transformation

E.coli MC1061 cells were made competent for transformation by growing a 100 ml culture to early log phase (A_{600}=0.3), pelleting the cells by centrifugation (5,000 rpm, JA-20 rotor, 5 min.), resuspending in 5 ml of 100 mM $MgCl_2$, followed by respinning and resuspending in 2.5 ml of ice cold 100 mM $CaCl_2$. The cells were then incubated on ice for one hour.

To acheive transformation the ligation reaction was combined with 200 ul of competent cells and incubated on ice for 40 minutes. The cells were then heat shocked at 42°C for 2 minutes, followed by incubation on ice for a further 5 minutes. 1 ml of L-broth was then added and the cells incubated with shaking at 37°C for 30 minutes. Aliquots were then plated onto L-agar plates containing 100 ug/ml ampicillin and incubated at 37°C overnight. Approximately 1000 colonies were obtained per cDNA ligation.

6.3.4 Detection and analysis of recombinant plasmid and cosmid clones

i) Screening bacterial colonies (Grunstein and Hogness, 1975)

Colonies to be screened with radioactive probes were toothpicked to a master plate (L-agar) and also to a plate overlayed with a pre-boiled nitrocellulose disc; both plates contained ampicillin. The master plate was incubated at 37°C overnight while the nitrocellulose plate was incubated at 37°C only until small colonies were visible (approximately 4 hours) then the nitrocellulose disc was transferred to an L-agar plate containing chloramphenicol (170 ug/ml) and incubated at 37°C overnight.

The master plate was stored at 4°C. The bacterial colonies on the

nitrocellulose disc were lysed by transferring the disc sequentially onto Whatman 3MM paper saturated with 10% (w/v) SDS for 5 minutes, then 0.5 M NaOH, 1.5 M NaCl for 5 minutes, followed by 0.5 M Tris-HCl pH7.5, 1.5 M NaCl for 5 minutes. The discs were then baked for 2 hours at 80 ^{o}C _in vacuo_. Hybridization and washing conditions were as described previously for Southerns (6.3.2.iv).

ii) _Minisreen examination of plasmid recombinants_

Small scale plasmid isolations from potential recombinants were done by the procedure of Birnboim and Doly (1979).

1.5 ml cultures of recombinants were grown overnight in L-broth plus antibiotic. The cells were pelleted by centrifugation for one minute in an Eppendorf centrifuge then resuspended in 100 ul of 15% (w/v) sucrose, 25 mM Tris-HCl pH8.0, 5 mM EDTA, 1 mg/ml lysozyme and incubated on ice for 10 minutes. 200 ul of 0.2 M NaOH, 1% (w/v) SDS was then added and the solution returned to ice for 5 minutes. 125 ul of ice cold 3 M Na-acetate pH4.6 was then added, gently mixed and incubated on ice for 10 minutes.

Insoluble material was removed by centrifugation (10 min., Eppendorf centrifuge) and the supernatant phenol/chloroform extracted. The aqueous phase was recovered and the DNA ethanol precipitated. The pellet was resuspended in 200 ul of TE buffer then digested with 50 ug/ml RNase A for 30 minutes at 37 ^{o}C. After phenol/chloroform extraction the plasmid DNA was collected by ethanol precipitation and resuspended in TE buffer.

iii) _Subcloning into plasmids_

Vector preparation and ligation

Vector DNA was prepared by digesting the plasmid of choice with a 5-fold excess of restriction enzyme in a 20 ul reaction (6.3.2.i). The 5' terminal phosphates were then removed by adding 2 ul of 1 M Tris-HCl pH8.0 and 1 ul of 0.1U/ul CIP and incubating at 37 ^{o}C for 60 minutes. The phos-

phatased vector was separated from contaminating supercoils by purification through low melting temperature agarose (6.3.2.ii).

Restriction fragments to be subcloned were also isolated by electrophoresis through low melting temperature agarose (6.3.2.ii).

Vector and insert DNA were ligated in 10 ul reaction mixtures which contained 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and either 0.1U (sticky-end ligations) or 1U (blunt-end ligations) of T4 DNA ligase, and were incubated at room temperature for 1-4 hours. Reactions contained 10-20 ng of vector and enough insert to give a 2:1 vector to insert molar ratio.

Ligations were transformed into competent E.coli MC1061 cells as previously described (6.3.3.v).

Blunt-ending

Restriction fragments which generated unsuitable 5' or 3' overhangs were converted to blunt-ends to enable cloning into a blunt-ended vector. The fragment was incubated at 37 ^{o}C for 30 minutes in a 10 ul reaction mix containing 10 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 uM dNTPs and 1U of DNA polymerase I Klenow fragment. The reaction was terminated by phenol/chloroform extraction and the DNA recovered by ethanol precipitation.

6.3.5 Preparation of in vitro labelled DNA

i) Nick-translation (Maniatis et al., 1975)

Nick-translation of restriction fragments isolated from low melting temperature agarose gels (or of whole plasmid) were carried out in 20 ul reactions which were incubated at 14 ^{o}C for 90 minutes. Each reaction contained 100 ng of DNA, 50 uCi of α-^{32}P-dCTP (1800 Ci/mmole), 5 mM $MgCl_2$, 50 mM Tris-HCl pH7.6, 50 ug/ml gelatine, 0.1 mM DTT, 25 uM dATP, 25 uM dTTP, 25 uM dGTP, 5U of DNA polymerase I and 40 pg DNase I. If extremely high specific activities were required the amount of cold dATP in the

reaction was dropped to 0.2 uM and 25 uCi of α-^{32}P-dATP (1800 Ci/mmole) was included.

Unincorporated nucleotides were separated from the labelled DNA by chromatography through Sephadex G-50 (fine) equilibrated in TE buffer. The 20 ul reaction was directly applied to a 1.5 ml G-50 column in a Pasteur pipette and washed through with 200 ul aliquots of TE. The first radioactive peak to come through the column was collected and a 1 ul sample removed and assayed by Cherenkov counting. Specific activities of between 5×10^7 and 2×10^8 cpm/ug were obtained using 50 uCi of α-^{32}P-dCTP and activities of between 2×10^8 and 5×10^8 cpm/ug were obtained when 50 uCi of α-^{32}P-dCTP and 25 uCi of α-^{32}P-dATP were used.

ii) <u>Kinasing</u>

Oligonucleotides were end-labelled at their 5' termini using T4 polynucleotide kinase (PNK). Kinasing was done in 10 ul reactions which contained 50 uCi γ-^{32}P-ATP (2000 Ci/mmole), 200 ng oligonucleotide, 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM DTT and 1U of PNK, and were incubated at 37 ^{o}C for 60 minutes.

Unincorporated label was separated from kinased oligonucleotides by electrophoresis through a 20% (w/v) polyacrylamide gel (6.3.2.iii). The band corresponding to labelled oligonucleotide was located by autoradiography, excised with a scalpel and eluted by incubation at 70 ^{o}C for one hour in 400 ul of TE.

6.3.6 <u>Cloning restriction fragments into M13</u>

i) <u>Preparation of double-stranded replicative form (RF) DNA</u>

A plaque from a plate stock of M13 'phage was picked into 1 ml of 2 X YT broth (6.2.4.ii) and grown with shaking at 37 ^{o}C for 5 hours. This , plus 10 ml of an overnight culture of <u>E.coli</u> JM101 grown in minimal

media (6.2.4.ii), were poured into 500 ml of 2 X YT broth and incubated at $37^{o}C$ for 4 hours with shaking. After this time the cells were collected by centrifugation (6,000 rpm, JA-12 rotor, 10 min., $4^{o}C$) and the double stranded RF DNA isolated by the CsCl procedure described for plasmids and cosmids (6.3.1.i).

ii) BAL-31 digestion of DNA

BAL-31 exonuclease was used to generate M13 deletion derivatives for dideoxy sequencing (6.3.7). The DNA to be digested, usually 2-3 ug, was linearized with the appropriate restriction enzyme then phenol/chloroform extracted and ethanol precipitated. This linear DNA was digested in a 25 ul reaction which contained 600 mM NaCl, 20 mM Tris-HCl pH8.0, 12 mM $CaCl_2$, 12 mM $MgCl_2$ and 1 mM EDTA. The reaction was started by adding 1U of BAL-31 and the sample incubated at $30^{o}C$. Aliquots were removed at various times and the reaction terminated by phenol/chloroform extraction then the DNA collected by ethanol precipitation. The BAL-31 treated DNA was then blunt-ended in a 10 ul Klenow reaction (6.3.4.iii) at $37^{o}C$ for 15 minutes. After secondary restriction enzyme digestion the DNA fragments were fractionated and purified from low melting temperature agarose (6.3.2.ii).

iii) Ligation and transformation

DNA fragments were cloned into the M13 vectors mp18 and mp19 by the same procedure as described for subcloning into plasmid vectors (6.3.4.iii). Ligations contained 20 ng of dephosphorylated M13 vector and enough insert to give a 3:1 molar ratio of vector to insert.

Ligations were transformed into competent *E.coli* JM101 cells which were prepared by growing JM101 cells in 2 X YT broth to an A_{600} of 0.3, collecting the cells by centrifugation (JA-20 rotor, 6,000 rpm, 5 min.), and resuspending in 2.5 ml of fresh 50 mM $CaCl_2$ followed by incubation on ice for at least 2 hours. The ligation was added to 200 ul of competent

cells and left on ice for 40 minutes. The cells were heat shocked at 42 °C for 2 minutes then added to 3 ml of molten 0.7% (w/v) agar containing 20 ul of 10 mg/ml BCIG, 20 ul of 10 mg/ml IPTG and 20 ul of a JM101 overnight culture. This mixture was plated onto minimal agar plates and grown at 37 °C overnight. Recombinant plaques were identified by the loss of blue plaque phenotype due to insertional inactivation.

iv) Preparation of single-stranded M13 recombinant DNA

Recombinant plaques were toothpicked into 1 ml of 2 X YT broth containing 25 ul of an overnight culture of JM101 (grown in minimal media) and grown with shaking at 37 °C for 5 hours. Cells were pelleted by spinning in an Eppendorf centrifuge for 5 minutes. The supernatant was transferred to a fresh tube which contained 200 ul of 2.5 M NaCl, 20% (w/v) PEG 6000, mixed well and incubated at room temperature for 15 minutes, after which time the 'phage were collected by centrifugation (Eppendorf, 4 °C, 10 min.). After removal of all the supernatant the 'phage pellet was resuspended in 100 ul of TE and extracted firstly with phenol (100 ul) then with diethyl-ether (200 ul). The purified 'phage DNA was then collected by ethanol precipitation, resuspended in 25 ul of TE and stored at -20 °C.

6.3.7 Dideoxy sequencing (Sanger *et al.*, 1977)

i) Hybridization

Either 2.5 ng of universal sequencing primer (USP, 6.2.5) or 5 ng of PGH gene-specific primer (6.2.5) was annealed to approximately 500 ng of M13 single-stranded template (5 ul of a 25 ul single-stranded DNA preparation (6.3.6.iv)) in a 10 ul volume also containing 10 mM Tris-HCl pH7.8, 10 mM $MgCl_2$. This solution was heated to 70 °C for 5 minutes then cooled at room temperature for 5 minutes.

ii) Polymerization

The hybridization mix was added to 10 uCi of dried down α - ^{32}P-dATP, vortexed to resuspend the label and then 0.5 ul of 3U/ul DNA polymerase I Klenow fragment added. 2 ul of this solution was dispensed into each of four tubes which already contained 2 ul of the appropriate dNTP/ddNTP mix. The dNTP/ddNTP mixes contained the following ; T mix: 10 uM dTTP, 200 uM dCTP, 200 uM dGTP, 0.5 mM ddTTP ; C mix: 200 uM dTTP, 10 uM dCTP, 200 uM dGTP and 0.2 mM ddCTP ; G mix: 200 uM dTTP, 200 uM dCTP, 10 uM dGTP and 0.2 mM ddGTP ; A mix: 200 uM dTTP, 200 uM dCTP, 200 uM dGTP and 50 uM ddATP (each mix also contained 5 mM Tris-HCl pH8.0 and 0.1 mM EDTA). After adding the 2 ul of hybridization mix the solutions were mixed by a 1 minute centrifugation (Eppendorf), then incubated at 37 ^{o}C for 10 minutes. 1 ul of 0.5 mM dATP chase was added to each tube and after centrifugation incubation was continued for a further 10 minutes at 37 ^{o}C.

3 ul of formamide loading buffer (95% formamide (v/v), 20 mM EDTA pH8.0, 0.1% (w/v) each of bromocresol purple and xylene cyanol) was then added to each tube to stop the reactions, and after mixing the reactions were boiled for 3 minutes.

iii) Sequencing gels

1 ul aliquots of each reaction were analysed on 6% acrylamide/8 M urea gels which were polymerized and run in 1 X TBE buffer (6.3.2.iii). Gels were pre-electrophoresed at 20 mA for 20 minutes before use, and run at 30 mA constant current. Following electrophoresis gels were fixed for 10 minutes with 10% (v/v) acetic acid, washed with 4 litres of 20% (v/v) ethanol, then baked dry. Autoradiography was carried out at room temperature for 3 to 16 hours.

6.3.8 Oligonucleotide directed mutagenesis (Zoller and Smith, 1983)

i) Kinasing oligonucleotides

The oligonucleotide primers used in mutagenesis reactions were all kinased before use. 10 ul kinasing reactions contained 100 ng of oligonucleotide, 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1U of PNK and were incubated at 37 ^{o}C for 1 hour, then heated to 70 ^{o}C for 5 minutes, diluted to 4 ng/ul and stored at -20 ^{o}C.

ii) Extension and transformation

Each mutagenesis reaction contained two oligonucleotides, both of which were kinased. In addition to the mutagenesis oligonucleotide, the USP 17-mer (6.2.5) was included in each reaction. Oligonucleotides were annealed to single stranded M13 templates by combining 1 ul of M13 DNA (from a 1 ml single-stranded preparation in 25 ul (6.3.6.iv)), 1 ul of mutagenesis oligonucleotide (4 ng/ul), 2 ul of USP (2 ng/ul), 5 ul of 200 mM NaCl and 5 ul of 10 X TM (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$). These solutions were mixed, heated to 70 ^{o}C for 5 minutes and then cooled at room temperature for 5 minutes. 5 ul of 500 uM dNTP mix (500 uM of each dNTP), 5 ul of 10 mM ATP, 5 ul of 10 mM DTT, 2 ul of 3U/ul DNA polymerase I Klenow fragment and 1 ul of 1 U/ul T4 DNA ligase were then added and after gentle mixing incubated at room temperature for 4-16 hours. Aliquots of 0.5, 1 and 2 ul of the reaction were then transformed into competent JM101 cells (6.3.6.iii).

iii) Screening plaques for mutants

Plates containing M13 plaques were chilled at 4 ^{o}C for 30 minutes after overnight growth at 37 ^{o}C. A plate containing approximately 100 well separated plaques was chosen for screening. Duplicate nitrocellulose lifts of plates were made by the method of Benton and Davis (1977). After air drying the filters were baked without any further treatment at 80 ^{o}C in

vacuo for 2 hours. Filters were prehybridized for 2 hours at 42°C in a solution containing 0.9 M NaCl, 90 mM Tris-HCl pH7.6, 6 mM EDTA, 5 X Denhardts, 100 ug/ml sonicated salmon sperm DNA and 0.5% (v/v) Nonidet P40. Following prehybridization mutagenesis oligonucleotide which had been ^{32}P-labelled by kinasing (6.3.5.ii) was added to a concentration of 10 ng/ml and incubation with shaking at 42°C continued overnight.

Washing discs with TMACl

Mutants were distinguished from parental plaques by selective washing in 3 M tetramethylammonium chloride (TMACl). In a 3 M solution of this compound G/C and A/T base pairs have equal binding energies allowing base independent melting temperatures which can be estimated from a pre-determined melting curve (Wood *et al.*, 1985).

Following overnight hybridization at 42°C, discs were rinsed twice with 6 X SSC at room temperature then once with TMACl solution (contains 3M TMACl, 50 mM Tris-HCl pH8.0, 0.1% (w/v) SDS and 1 mM EDTA) at room temperature. Two 30 minute washes with TMACl solution were then done at the selection temperature determined from the melting profile presented by Wood *et al.* (1985). The discs were then wrapped in plastic wrap and autoradiographed in the presence of an intensifying screen for between 4 and 20 hours at -80°C .

The percentage of positive plaques varied greatly in different experiments, and seemed to be due entirely to the specific mutagenesis primer used. Efficiencies of between 15 and 35% were typical.

6.3.9 Isolation and analysis of RNA

i) Isolation of RNA from E.coli

RNA was isolated from 5 ml overnight cultures of *E.coli*. Cells were pelleted (JA-20, 6,000 rpm, 10 min.) and then lysis performed by resuspend-

ing in 4 ml of 10 mM Tris-HCl pH8.0, 1 mM EDTA, 1 mg/ml lysozyme followed by incubation on ice for 5 minutes. Three cycles of freeze/thawing (-80 ^{o}C to 37 ^{o}C) were then carried out. An equal volume of 7.5 M CsCl was then added and the lysate poured into a 10 ml SW 41 centrifuge tube. A 2.5 ml pad of 5.8 M CsCl was then added and the tubes spun in a SW 41 rotor at 30,000 rpm for 20 hours at 20 ^{o}C. The supernatant was then poured off and the RNA resuspended in 500 ul of H_2O. The RNA was then phenol/chloroform extracted, ethanol precipitated and resuspended in 100 ul of H_2O. RNA was stored at -20 ^{o}C in 70 % (v/v) ethanol.

ii) Small scale isolation of RNA from animal tissue

Small scale RNA isolations from animal tissue were performed by grinding the tissue in liquid nitrogen, and resuspending in 4 mls of a solution containing 4 M guanidine isothiocyanate, 1 % (w/v) sarkosyl, 100 mM ß-mercaptoethanol and 50 mM Tris-HCl pH7.5. This solution was drawn through an 18 gauge syringe to shear the DNA, then mixed with an equal volume of 7.5 M CsCl. This solution was poured into an SW-41 centrifuge tube and a 2 ml 5.8 M CsCl pad added, and the tubes spun and RNA collected as described above (6.3.9.i).

iii) Electrophoresis of RNA and Northern blotting (Thomas, 1980)

RNA samples were denatured with glyoxal before electrophoresis through agarose gels. RNA was precipitated out of 70% ethanol, vacuum dried, and resuspended in 5 ul of formamide. After incubation at 80 ^{o}C for 5 minutes, 1.5 ul of 6 M deionized glyoxal and 4 ul of 20 mM Na-phosphate pH6.5 was added and the sample incubated at 50 ^{o}C for 15 minutes. 5 ul of load buffer (50% (v/v) glycerol, 10 mM Na-phosphate pH6.5, 0.1% (w/v) bromocresol purple) was then added and the sample electrophoresed through a 1.5% agarose gel run in 10 mM Na-phosphate pH6.5. RNA was visualized by staining with ethidium bromide (gels which were blotted were not stained).

RNA was transferred onto nitrocellulose filters ("Northern blots") without any further treatment by the method of Southern (1975) using 20 X SSC as the transfer buffer. Alternately, RNA was transferred to Gene-screen nylon membranes using 10 mM Na-phosphate pH6.5 as the transfer buffer. Hybridization, washing and autoradiography were done as previously described (6.3.2.iv) .

iv) Primer extension (McKnight et al., 1981)

Primer extension products were generated for analysis by annealing a ^{32}P-end-labelled oligonucleotide to the RNA of interest and synthesizing a complementary run-off strand with reverse transcriptase.

Oligonucleotides were end- labelled as described (6.3.5.ii) and annealed to RNA (0.5 to 20 ug) in a 10 ul reaction which also contained 100 mM KCl and 100 mM Tris-HCl pH8.3 and was heated to 70°C for 5 minutes then cooled at room temperature for 15 minutes. The reaction volume was then increased to 20 ul by the addition of all four dNTPs to a final concentration of 500 uM each, DTT to 10 mM, $MgCl_2$ to 10 mM and 10 U of reverse transcriptase. The reaction was incubated at 42°C for 60 minutes then RNase A added to a final concentration of 50 ug/ml and the reaction incubated at 37°C for 15 minutes. The reaction was stopped by phenol/chloroform extraction and the nucleic acids collected by ethanol precipitation. Extension products were resuspended in 5 ul of formamide loading buffer, boiled for 5 minutes then electrophoresed on a 6 or 12% acrylamide gel containing 8 M urea (6.3.7.iii). Extension products were visualized by autoradiography at -80°C in the presence of an intensifying screen.

6.3.10 Preparation and analysis of proteins

i) Preparation of protein from E.coli

Protein extracts were prepared from overnight cultures of E.coli cells by collecting the cells (Eppendorf centrifuge, room temp., 2 min.)

and resuspending in a one tenth volume of 2% (w/v) SDS, 1 M ß-mercaptoethanol. After dispersion by vortexing the cells were boiled for 5 minutes. Extracts were stored at $-20^{o}C$.

ii) SDS gel electrophoresis (Laemmli, 1970)

Bacterial protein extracts were electrophoresed on 12.5% acrylamide (38:1 acrylamide:bis-acrylamide) gels (120 X 120 X 0.9 mm), with a 2 cm 4% acrylamide stacking gel. Gels were poured in 1 X gel buffer which contained 375 mM Tris-HCl pH8.8, 0.1% SDS and were run in running buffer which contained 250 mM Tris base, 200 mM glycine and 0.1% (w/v) SDS.

An equal volume of 2 X SDS load (10% (v/v) glycerol, 0.1% (w/v) SDS, 375 mM Tris-HCl pH8.8, 5% (v/v) ß-mercaptoethanol) was added to samples which were then boiled for 4 minutes before loading. Electrophoresis was carried out at 25 mA until the marker dye reached the gel bottom. Gels were stained in 0.1% (w/v) coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid at $65^{o}C$ for 60 minutes then destained in a number of changes of 5% (v/v) methanol, 10% (v/v) acetic acid. Usually 5 ul of bacterial extract was run per track.

6.3.11 Generation of transgenic animals (performed by A.Michalska)

i) Transgenic mice

One-cell *in vivo* fertilized embryos were collected from superovulated C57xCBA female mice 21 to 23 hours after hCG injection. Approximately 600 copies of the linear plasmid insert (1pl of a 2ng/ul solution in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4)) were injected into the male pro-nuclei of the one cell embryos using a Zeiss microscope and 300x magnification. Following injection the zygotes were surgically transferred to the oviducts of pseudopregnant C57xCBA F1 mice.

ii) Transgenic pigs

One-cell pig embryos which had been fertilized *in vivo* were collected

from large white sows 5 hours after mating. The pronuclei were visualized by centrifuging eggs at 7000 X g for 3 minutes, and one of the pronuclei injected with 1pl of PBS containing approximately 600 copies of the linear plasmid insert. Following overnight culture, 30 microinjected embryos were surgically transferred to the oviducts of synchronized recipient large white sows. Approximately 20 % of the implanted sows became pregnant, and farrowed small litters of 4 to 5 piglets.

6.3.12 Containment facilities

All manipulations involving recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

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