CONTROLLED PRODUCTION OF GROWTH HORMONE AND FERTILITY IN TRANSGENIC RATS

by

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Abstract

This study aimed to develop a transgenic rat model to allow the study of the effects of GH expression , growth regulation and reproductive function. Transgenic rats were created by pronuclear microinjection of a human metallothionein promoter porcine growth hormone (pGH) gene construct. Of the 546 eggs injected and transferred, 70 young were born, of which 39 were shown to be transgenic, which equalled an integration frequency of 56 percent.

Eleven of the 39 transgenic offspring produced pGH in their blood at the time of weaning at plasma concentrations between 18 ng/ml-1200 ng/ml. Nine of the 11 animals with plasma pGH levels of >42 ng/ml at weaning exhibited enhanced growth relative to their non-transgenic littermates, whereas the other two animals exhibited apparently normal growth. Two breeding lines (D, M) were established from transgenic females. Animals from Line M differed from Line D in exhibiting high levels of plasma pGH and accelerated growth, whereas Line D offspring showed low levels of circulating pGH and normal growth. However, in Line D animals further expression of the pGH transgene and growth was inducible by zinc treatment. The different patterns of pGH expression and growth phenotypes in the both lines were shown to be stably co-inherited with the transgene for 5 generations in the D line and 7 generations in the M line. Examination of the tissue-site of pGH expression showed predominance in the liver in Line M and the intestine in Line D.

In contrast to previous studies with transgenic mice, females rats from both lines which were heterozygous for the transgene showed enhanced fertility which was apparently related to an increase in ovulation rate. No impairments were found in the length of oestrous cycle, period of

matings or pregnancy rate. Furthermore, during lactation, milk production was enhanced in transgenic animals compared to that in non-transgenic controls resulting in increased growth of the pups.

The study concluded that transgenesis can be used as a means of regulation of growth in the rat without the adverse side-effects on fertility as seen in transgenic mice; indeed, expression of pGH in transgenic rats can actively enhance female fertility probably due to a synergistic effect of GH in promoting the action of FSH on the ovary.

DECLARATION

I hereby declare that this thesis contains no material which has been submitted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person, except where due reference is made in the text.

If accepted for the award of a Ph.D. degree I consent to this thesis being available for loan and photocopying.

Z.T. Du

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PUBLICATIONS

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ABREVIATION

Chemicals

BSA	bovine serum albumin
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
HCO_3	bicarbonate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
PBS	Phosphate buffered saline
Tris	2-amino-2-hydroxymethyl-propane-1-3-diol

Hormones

E_2	oestradiol-17β
FSH	follicle stimulating hormone
GH(s)	growth hormone(s) (bovine, human, ovine, porcine and rat)
GHBP	growth hormone binding protein
GRF	growth hormone-release factor
IGF	insulin-like growth factor
IGFBP's	insulin-like growth factor binding proteins
hCG	human chorionic gonadotropin
LH	luteinizing hormone
P	progesterone
PMSG	pregnant mare serum gonadotropin
PRL	prolactin

Symbols and units

Ci	curie
cm	centimetre(s)
g	gram(s)
h	hour(s)

i.u.	international unit(s)
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
%	percent
<	less than
=	equal to
>	greater than
kg	kilogram(s)
l	litre
M	molar
mg	milligram(s)
ml	millilitre(s)
mM	millimole(s)
mm	millimetre(s)
mOsm	milliosmolar
ng	nanogram(s)
°C	degrees Centigrade
pl	picolitre(s)

Others

BW	body weight
BSA	bovine serum albumin
cpm	counts per minute
e.g.	for example
ES	embryonic stem cell
et al	et alia (and others)
ICM	inner cell mass
ID	inner diameter
mRNA	messenger RNA

p(plural pp)	pages
PCR	polymerase chain reaction
RNA	ribonucleic acid
RPA	ribonuclease protection analysis
UV	ultraviolet light
v/v	volume:volume
vs	versus
w/v	weight:volume
xg	unit of gravitational field

Chapter 1
Introduction

1.1 INTRODUCTION

The term transgenesis was coined by Gordon and Ruddle (1983) to describe a technical process enabling transfer of inheritable functional genes between organisms irrespective of species barriers. Recent usage has extended its meaning to include not only gene transfer, but manipulation of resident genes to allow over and under-expression in designated tissues at different stages of development. Transgenic animals are now produced as a matter of routine in many laboratories throughout the world and have been instrumental in providing new insights into mechanisms of development and developmental gene regulation, into the action of oncogenes, and into the intricate matrix of cellular interactions within the immune system. Furthermore, the transgenic technology offers exciting possibilities for generating precise animal models for human genetic diseases and for producing large quantities of economically important proteins by means of genetically engineered farm animals (see reviews, Palmiter and Brinster, 1986; Jaenisch, 1988; Pursel *et al.*, 1989; Gordon, 1989; Massey, 1990; Pursel *et al.*, 1990; 1990a; First, 1990; Seamark, 1991; Seamark and Wells, 1993).

One of the first questions investigated by transgenic technology was the regulation of growth (Palmiter *et al.*, 1982). The general strategy in this study was to introduce growth-regulating genes, under the control of heterologous promoters, into the germ line to allow long-term controlled production of growth factors in specified tissues, thus creating a system in which the effects of these factors can be assessed in an otherwise normal animal.

These studies, mostly carried out using transgenic mice, have yielded valuable insight into growth processes. However, the regulation of postnatal growth is exceedingly complex and involves an interplay between circulating growth promoting hormones, genetic potential, and the prevailing nutritional status of the animal (Pursel *et al.*, 1989). Transgenic mice carrying introduced foreign growth factor genes, including growth hormone and insulin like growth factors, have been commonly available for more than ten years. However, this model has limitations relating to the size of mice and the difficulties in blood collection and other measurement which have been a major constraint in some studies.

This thesis is concerned with an attempt to develop a transgenic rat model to overcome these size limitations and to further our understanding of the mechanism of growth regulation and specifically to study the adverse effects of growth hormone (GH) on fertility shown in a mouse model. The small size of the mouse prevented repeated blood sampling and other *in vivo* experiments required for detailed studies of the cause of the GH induced infertility. The rat was therefore chosen as being a suitable alternative as it resembles the mouse in important reproductive features such as high fecundity, non-seasonal breeding, short oestrus cycle and generation time, but being larger, has a size advantage over mice which allows repeated blood sampling and experimental protocols which are less easily performed, or precluded with mice.

1.2 GENE TRANSFER

The development of a technology which allows the introduction of novel genes into the germ line of mammals was a major advance in biological research methodologies, especially in the application in studies of gene function in diploid organisms where their long life cycle made classical genetic approaches towards the understanding of growth regulation impractical (see review, Palmiter & Brinster 1986).

1.2.1 Methodologies

In the last 20 years a variety of methods for introducing foreign DNA into both somatic and germ cells of mammals have been developed, some of which have developed into routine procedures with application world wide. These systems include microinjection of DNA into single cell embryos, the infection of early embryos with retroviruses and embryonic stem cell transfer into blastocysts (see reviews, Gordon & Ruddle, 1985; Palmiter and Brinster, 1986; Jaenisch, 1988; Pursel *et al.*, 1989; Seamark, 1989; first, 1990; Massey, 1990; Seamark, 1991; Seamark and Wells, 1993).

1.2.1.1 Pronuclear microinjection

The most common procedure used for gene transfer is to microinject the foreign DNA into one of the pronuclei of a recently developed zygote which is then transferred to a surrogate mother for rearing. The principal advantage of microinjection of recombinant DNA into the pronucleus is the efficiency of generating transgenic lines which express the transgene in a predictable manner. During the last 15

years, since the first successful application of this approach in mice (Gordon *et al.*, 1980; Brinster *et al.*, 1981; Constantini & Lacy, 1981; Harbers *et al.*, 1981; Wagner *et al.*, 1981), the microinjection approach has been used extensively in transgenesis resulting in the production of thousands of transgenic mice carrying an array of transgenes (see reviews, Palmiter and Brinster, 1986; Jaenisch, 1988; Brem *et al.*, 1989; Wanke *et al.*, 1991; Bartke *et al.*, 1994). The technique has also been successfully adapted to other species including sheep (Pinkert *et al.*, 1987; Pursel *et al.*, 1987; Simons *et al.*, 1988; Murray *et al.*, 1989), pigs (Hammer *et al.*, 1985; Ebert *et al.*, 1988; Pursel, 1987; Vize *et al.*, 1988), cow (Biery *et al.*, 1988), goat (Armstrong *et al.*, 1987), rabbits (Hammer *et al.*, 1985) and rats (Hammer *et al.*, 1990; Mullins *et al.*, 1990; Hochi *et al.*, 1991, 1992) with both integration and expression of the transgenes (see recent reviews, Pursel *et al.*, 1989;.1990; Rexroad *et al.*, 1990; Massay, 1990; First, 1990; Seamark and Wells, 1993)

The basic technique of directly pronuclear microinjection involves impaling the pronucleus with a fine glass injection pipette and forcing a DNA solution out of the pipette into the pronucleus by pressure. Typically, 2 µm diameter pipettes fined to about 0.2 µm OD at the tip are used to inject about 2 picolitre DNA solution into the male pronucleus of one cell zygotes. A 90% embryo survival rate following microinjection can be achieved by this means. The methodology and results of this approach in recent studies have been reviewed extensively by several authors (Brinster & Palmiter, 1982; Palmiter & Brinster, 1986; Church *et al.*, 1985; Gordon & Ruddle , 1985; Gordon, 1989; Seamark, 1989; 1991; Seamark & Wells, 1993; Jaenisch, 1988; First, 1990; Rexroad, 1990).

However, one disadvantage of this method is that it is not readily adaptable to the introduction of genes into zygotes at later developmental stages.

1.2.1.2 Retrovirus infection

Infection of mouse embryos with retroviruses constitutes another method of genetically manipulating mouse embryos. The first animals carrying introduced foreign genes were derived by microinjection of simian virus 40 DNA into the blastocyst cavity (Jaenisch & Mintz, 1974). Integration of the viral DNA into the germ line was demonstrated in subsequent studies when mouse embryos were exposed to infectious moloney leukemia retrovirus, resulting in the generation of the first transgenic mouse strains (Jaenisch, 1976). This facile technique is currently receiving renewed attention because the retroviral DNA can be used to "a single copy of insert" and have it integrated with the chromosome. However, it has three severe drawbacks namely (a) the foreign DNA is limited in size, (b) founder animals are mosaics as infection occurs after cell division begins, and (c) the retroviral DNA sequences interfere with expression of the genes that it carries (Palmiter & Brinster, 1986).

1.2.1.3 Embryonic stem cell transfer

The approach receiving current attention is an indirect one in which the DNA is introduced into embryonic stem (ES) cells which are then incorporated into the inner cell mass (ICM) of host blastocysts by injection or through aggregation with precompaction embryos (Lovell-Badge *et al.*, 1985; Wagner *et al.*, 1985; Robertson, 1986). The

attraction of this approach is that the foreign DNA can be introduced into the cultured ES cells by any one of the variety of standard methods used by molecular biologists. The cells with the desirable genetic characteristics can be then selected for clonal expansion prior to reintroduction into an embryo. Conditions which allow ES cell lines to retain their full developmental properties are well established for the mouse (Robertson, 1986; 1987) and since 1987 have been widely employed in gene-targeting procedures (Doetschmann *et al.*, 1987; Thomas & Capecchi, 1987, Kuehn *et al.*, 1987). As a result there are now a wide range of germline modified mice lines available with applications to all branches of medical and biological research. Application of the technology to other species is still in its infancy but is developing quickly, driven by the distinct prospect of this procedure, making possible a revolution in animal breeding and marketing practices. Active study of the application of ES cell technology to all major livestock and laboratory species is now being carried out throughout the world.

1.2.2 Integration of transgene

A relatively high degree of integration of introduced genes has been obtained in the studies with hybrid or fusion genes in which the promoter/regulatory region was replaced with a heterologous one (Brinster *et al.*, 1985). It is thought that the injected DNA molecules associate by homologous recombination before integration and in most cases insert subsequently at a single chromosomal site (Jaenisch, 1988). It is thought that these sites occur at random chromosome breaks, possibly caused by repair enzymes activated by the free ends of the injected DNA molecules (Brinster *et al.*, 1985). Clearly,

rearrangements, deletions, duplications and translocations of the host sequences can occur at the integration site (Mark, 1985; Covarrublas, 1986; Mahon, 1988). However, because integration usually occurs prior to the first round of DNA replication, most transgenic mice carry the transgenes in all of their cells, including the germ cells. If integration occurs after one or more rounds of replication, this results in the transgenes being present in only a fraction of the cells with the mice usually showing the same degree of mosaicism in somatic and germ cells (see review, Palmiter and Brinster, 1986).

1.2.2.1 *Factors affecting integration*

Several parameters affect the efficiency of integration of foreign DNA into mice, as noted by Brinster and his associates (Brinster *et al.*, 1985):

1 The integration efficiency improved as the DNA concentration in the injection fluid increased to about 1 ng/µl. No further increase was seen above this concentration and increasingly poor embryo survival was observed.

2 DNA integration was slightly more efficient following injection into male compared with the female pronucleus.

3 There was a higher frequency of integration following microinjection of DNA into the eggs from hybrid compared with inbred donor mice.

4 The size of the transgene had no effect on the integration frequency up to 50 kb, but linearization of the DNA prior to microinjection led to a 5 fold increase in integration frequency.

5 The vehicle used for DNA transfer into embryos should only contain low concentrations of MgCl or EDTA as both are toxic at high concentrations.

1.2.2.2 Efficiency of integration

The frequency of integration is highly variable. Under the conditions described by Brinster *et al.* (1985) and using linear DNA microinjected into the male pronucleus of one cell fertilised eggs obtained from hybrid mice, about 15-30% of mice born are transgenic with a overall efficiency of producing live animals from injected and transferred eggs of approximately 3-5% (recently reviewed by Seamark & Wells, 1993; Pursel *et al.*, 1990; Gordon, 1989; Jaenisch, 1988; Brem *et al.*, 1989).

Microinjected pig ova represented by offspring at birth has varied from 4.0% to 11.7% (see reviews, Pursel *et al.*, 1990; 1990a; Seamark and Wells,1993). Expressed as a percentage of injected ova that resulted in transgenic pigs, the overall efficiency varied from 0.31 to 1.73 (Hammer *et al.*, 1985; Pursel *et al.*, 1989; 1990; 1990a; Vize *et al.*, 1988; Brem *et al.*, 1988; Polge *et al.*, 1989; Seamark and Wells, 1993), which was much lower than the efficiency following the transfer of the same fusion genes into mice.

Sheep appear to have an even lower success in frequency of integration of microinjected foreign DNA than either mice or pigs. Hammer *et al.* (1985) reported only one transgenic lamb in the first group of 73 lambs screened (1.3%) but in two subsequent experiments, in utilizing metallothionein bovine GH (MT- bGH) and MT-human GH

Releasing Factor fusion genes, other researchers found that 2 of 47 (4.2%) and 9 of 63 (14.2%) of the lambs and fetuses screened were transgenic (Rexroad and Pursel, 1988). Simons *et al.* (1988) obtained 6 transgenic lambs from 109 lambs born and Murray *et al.* (1989) reported that 7 of the 93 (7.5%) lambs screened for incorporation of the injected MTIa-ovine GH fusion gene were transgenic. From these reports, the integration frequency following microinjection into pronuclear of sheep embryos was 6.5% (25/385) of late-stage fetuses or lambs born, with the overall efficiency from injected and transferred eggs of approximately 0.1-2.34%. In a wool transgenesis program being conducted in South Australia, 12 of 173 lambs born were assessed to have incorporated the transgene and all of the 10 lambs tested expressed the transgene (two lambs were born dead and were not tested). Hence, in agreement with other studies approximately 1% of zygotes developed into transgenic animals (Powell *et al.*, 1994).

1.2.3 Gene expression

Normal development depends upon the differential expression of many genes in various cells that make up the organism and requires some genes to be expressed exclusively in one cell type with others being expressed in a few or in most cell types. Genes representative of each of these categories have been identified and shown to express appropriately when introduced into mice as transgenes (see reviews Palmiter and Brinster, 1986; Jaenisch, 1988; Gordon, 1989; Brem *et al.*, 1989).

1.3 GROWTH HORMONE AND REGULATION OF GROWTH

Growth is controlled by an array of hormones produced principally in the hypothalamus, pituitary gland, and peripheral tissue. GH, an intermediate in this cascade, is produced in somatotroph cells of the anterior pituitary (Daughaday, 1981) under the neuroendocrinal regulation of two hypothalamic peptides, somatostatin and growth hormone-releasing factor (GRF). Somatostatin inhibits the release of GH (Brazeau *et al.*, 1973), whereas GRF stimulates both GH synthesis and release (River *et al.*, 1982). Both somatostatin and GRF are released from the hypothalamus via the hypothalamic-hypophysial portal blood system in response to neurotransmitters (see review, Frohman and Jansson, 1986). Endogenous GH is released from the pituitary in pulsatile manner (Schalch and Reichlin, 1966; Takahashi *et al.*, 1981; Owens *et al.*, 1991) and transported to a wide variety of target tissues via the circulatory system.

One of the many specific effects exhibited by GH is stimulation of longitudional bone growth (Cheek and Hill, 1974). GH also has a wide range of metabolic actions and affects the utilization of protein, lipids and carbohydrates (Kostyo and Nutting, 1974; Goodman and Schwartz, 1974; Altszuler, 1974).

The growth of animals is also influenced by other hormones differently in male or female (see review, Daughaday *et al.*, 1975). Some of them, like corticosteroids and oestrogen antagonize GH action and their excess is associated with retardation of growth. Others, including insulin, androgens and thyroid hormone, act synergistically with GH and support normal growth.

1.3.1 Structure and action of growth hormone

GH belongs to a family of related protein hormones which include prolactin and placental lactogen (See review, Wallis, 1981). GH is formed by a single polypeptide chain of approximately 190 amino acids with two inter-chain disulphide bridge (Niall *et al.*, 1973). The hormone is synthesized as a precursor with a hydrophobic signal peptide which is removed during secretion from pituitary cells (Sussman *et al.*, 1976; Seeburg *et al.*, 1977).

GH has been purified and its amino acid sequence determined for a number of species, including human, bovine, ovine, porcine and equine (see review, Dayhoff, 1972). In addition, the primary structures of growth hormone from rat (Seeburg *et al.*, 1977), mouse (Linzer and Talamantes, 1985), chicken (Souza *et al.*, 1984) and salmon (Sekine *et al.*, 1985) have been deduced from the nucleotide sequences of cDNA clones.

The central importance of the pituitary to growth regulation was first demonstrated by Evans and Long (1921) when they produced giants rats by intraperitoneal injections of anterior pituitary extracts. Several years later, Smith (1927) confirmed that the growth inhibition following hypophysectomy in rats could be offset by treatment with crude preparations of GH.

The first mammalian GH was isolated and purified from bovine pituitary more than 50 years ago (Li and Evans, 1944). Information of physiological effects of GH in animals has advanced rapidly since then

(see review, Smith *et al.*, 1955). The failure of animals to respond to GH of different species and the discovery that monkey, but not bovine or porcine GH, promotes nitrogen retention in hypophysectomized monkeys established the concept of species specificity of GH (see review, Friesen, 1980). This concept was later confirmed in studies with GH receptor which demonstrated that the binding site of the human GH (hGH) receptor is unique and recognizes only primate GH (Carr and Friesen, 1976; Lesniak *et al.*, 1977). Purified hGH was firstly isolated in 1956 (Li and Papkoff, 1956; Raben, 1956) and used clinically two years later to successfully treat hypopituitary dwarfism (Raben, 1958). The importance of GH in human growth was later corroborated by measurement of serum GH levels of patients with growth disorders. Acromegaly and dwarfism were found to be due to elevated and deficient GH secretion respectively (see review, Franchimont and Burger, 1975).

It is generally accepted that GH regulates metabolism by influencing protein, carbohydrate and lipid metabolism (Kostyo and Nutting, 1974; Altszuler, 1974; Goodman and Schwartz, 1974). In tissue culture, GH was found to enhance protein synthesis by directly affecting amino acid transport, ribosome numbers, mRNA and enzymatic apparatus involved in protein synthesis (Kostyo and Isaksson, 1977; Daughaday, 1981). The effects of GH on cell and somatic growth have been studied extensively (see review, Cheek and Hill, 1974). Growth of tissues depends on increases in cell number, size of the individual cells, or both. It has been demonstrated that GH stimulates growth of cartilage and other tissues by increasing the number of cells rather than by increasing cell size (Cheek, 1966; Beach and Kostyo, 1968; Goldspink and Goldberg, 1975).

In recent years, with the development of recombinant DNA technology, GHs have become available in a recombinant form from many species and the hormones derived by this means have been shown to be equally efficient in growth stimulation as those hormones isolated directly from pituitary tissues (Olson *et al.*, 1981; Hintz *et al.*, 1982; Kaplan *et al.*, 1986).

1.3.2 Alteration of growth by growth hormone treatment

The effects of exogenous GH on animal growth have been known for 60 years. The stimulatory effect of heterologous GHs on growth in hypophysectomized and mature female rats has been well documented (Evans *et al.*, 1948; Simpson *et al.*, 1949; Bates *et al.*, 1964; Emerson, 1973). GH therapy, using pituitary or recombinant hormones, is now used routinely in treating growth hormone deficiency in man (see review, Frasier, 1983).

Due to the scarcity of the purified GH, studies of the effect of exogenous GH on farm animals have only recently been carried out. Machlin (1972) reported that daily injections of porcine GH (pGH) significantly improved growth performance in young pigs by increasing daily weight gain and feed efficiency. Further studies from the pig indicate that treatment with either pituitary or recombinant pGH results in increased growth rates, feed conversion efficiency, free fatty acids, glucose, insulin and decreased adipose tissue and blood urea nitrogen with no adverse effects (Chung *et al.*, 1985; Etherton *et al.*, 1986; 1987; Boyd *et al.*, 1986; McLaren *et al.*, 1987). There is also additional body water as a result of increased protein and decreased adipose tissue (Campbell *et al.*, 1989). This effect has also been

observed in sheep (Johnson *et al.*, 1985) and cows (Bauman *et al.*, 1985). By using purified rat GH (rGH), Groesbeck *et al.* (1987) demonstrated that injection of large doses of rGH to prepubertal rats markedly accelerated body weight gain and skeletal growth, beyond the normal rate of growth.

Recently, the availability of large quantities of human and animal GH through recombinant-DNA technology has made study of both human and animal applications possible. Clinical trials carried out over the past few years established that recombinant human GH is equally potent in stimulating growth as pituitary-derived GH (Kaplan *et al.*, 1986) and in livestock, injections of recombinant bovine GH (bGH) to dairy cows showed it was as efficacious as the pituitary product in stimulating growth and milk production (Bauman, *et al.*, 1985). Similarly, the growth of salmon has been shown to be stimulated by salmon recombinant GH (Sekine *et al.*, 1985).

Growth rate is dependant on dose of GH administered (Evock *et al.*, 1988; Boyd *et al.*, 1986). Too large a dose of GH is potentially lethal and can cause unacceptable morbidity. For example, a large dose of GH decreases pig motility as a result of osteochrondrosis (Etherton *et al.*, 1987) and deaths from toxicity occurred at the end of the 20-24 day treatment of rats with large doses of rGH (Groesbeck *et al.*, 1987). Thus, before any clinical or agricultural applications it is imperative to determine the maximal doses of GH that can be used to regulate growth without adverse, ethically unacceptable consequences (Etherton *et al.*, 1987). In the rat, the dose of homologous GH required to induce a supranormal growth rate in prepubertal rats is very large, representing 68 mg/kg body weight (BW) and 13.6 mg/kg BW day, initially

(Groesbeck *et al.*, 1987). In contrast, similar increases in BW gain in hypophysectomized rats and mature females (30 weeks old) were elicited by smaller daily doses of rGH, as low as 0.4 mg/kg and 1.05 mg/kg BW day, respectively (Groesbeck *et al.*, 1987).

Growing pigs respond to pGH administration by an increase in live weight gain (Chung *et al.*, 1985; Etherton *et al.*, 1986) with the response being modified by factors such as sex, genotype, GH dosage and source and protein amino acid intake (Campbell *et al.*, 1988). Interestingly, the stimulating effects of rGH on growth rate are sustained following cessation of hormone treatment in prepubertal and mature female rats (Groesbeck *et al.*, 1987). Similar observations have been made to the pGH in growing pigs with a larger growth response occurring in females than males (Campbell *et al.*, 1989). Changes in growth by pGH are mediated at least in part via stimulation of muscle protein deposition with the magnitude of the pGH-response depending on the pigs intrinsic capacity for protein growth (Campbell *et al.*, 1989).

Pituitary rGH concentration in rGH-treated prepubertal rats is markedly reduced. Mean pituitary rGH concentration was reduced from 18.3 μg/mg of pituitary in control rats to 10.6 and 7.7 μg/mg of pituitary in rats treated for 20 days with 1 mg and 5 mg rGH/day (Groesbeck *et al.*, 1987). Analysis of lung, thyroid, adrenal, liver, kidney, spleen, myocardium, skeletal muscle and intestinal tissues after chronic treatment with GH, at a maximal efficient dose, indicated no deleterious effects upon the animal. The size of the somatotroph did not differ, but there was significantly fewer of them after GH treatment (Machlin, 1972).

1.3.3 IGF mediation

Despite intensive studies on the structure and function of GH, the mechanism of the hormone's stimulation of growth is still not fully understood. Early observations on the inability of GH to stimulate anabolic processes in cartilage from hypophysectomized rats, and the findings that normal serum or serum from GH-treated hypophysectomized rats, but not serum from untreated hypophysectomized ones, restored these anabolic processes led to formulation of the somatomedin hypothesis (Salmon and Daughaday, 1957). In this hypothesis GH is seen to stimulate somatomedin production by the liver (Daughaday *et al.*, 1972). Several somatomedins have been purified and one of these, somatomedin-C or insulin-like growth factor-I (IGF-I, Klapper *et al.*, 1983) as it is now known, has been shown to be the main mediator of the stimulatory effects of GH on postnatal somatic growth (Froesch, 1983; Herington *et al.*, 1983).

The correlation between IGF-I and GH was demonstrated by measuring IGF-I levels in the plasma of patients with growth hormone disorders. Acromegalics were found to have significantly higher levels of IGF-I than normal subjects, while GH-deficient patients had low or undetectable levels of IGF-I (Bala and Bhaumick, 1979). Administration of GH to pituitary dwarfs resulted in an increase in circulating IGF-I levels and in stimulation of linear growth (Copeland *et al.*, 1980). Low IGF-I levels observed in hypophysectomized animals were found to be alleviated by GH treatment (Kaufman *et al.*, 1978). IGF-I and IGF-II increase between 10 and 35 kgs in growing pigs. The total concentration of IGF-I and IGF-II in blood of growing pigs correlated positively with IGF binding protein (IGF-BP) activity. IGF-BP's are

believed to control the IGF-I and IGF-II levels in blood, at least in part, by mediating their rate of clearance from the circulation (Owens *et al.*, 1991). However, in growing pigs normal secreted endogenous GH did not appear to exert a significant immediate control over plasma IGF-I and IGF-II (Owens *et al.*, 1991).

IGF-I increases in plasma of prepubertal and mature rats and growing pigs treated with GH (Groesbeck *et al.*, 1987; Etherton, 1987; Owens, 1989). The maximum effective daily dosage of rat, ovine or bovine GH induced a significant, but not marked increase in IGF-I in rats (Chiang and Nicoll, 1991; Groesbeck *et al.*, 1987). At a mean post injection interval of 4.7 hours with rGH , serum rat IGF-I levels in prepubertal rats injected with 1 mg and 5 mg rGH/day had risen from 3.5 to 4.7 and 5.0 U/ml, respectively (Groesbeck *et al.*, 1987). Similar results were also achieved in pigs treated chronically with pGH (Sillence and Etherton, 1987). The independent actions of GH on protein and lipid metabolism may also be mediated via IGF-I, the release and synthesis of which appears to be inhibited by dietary protein deficiency in particular lysine (Campbell *et al.*, 1988).

The growth -promoting effects of IGF-I on cultured cells *in vitro,* smooth muscle cells and bone cells have been well documented (see review, Froesch *et al.*, 1985). Anabolic effects of IGF-I on body growth have been demonstrated in hypopituitary or otherwise GH-deficient rodents (Schoenle *et al.*, 1985; Skottner *et al.*, 1989), normal rats with free access to food (Philipps *et al.*, 1988), diabetic rats (Scheiwiler *et al.*, 1986) and fasted rats (Asakawa *et al.*, 1988). It was also shown that infusion of pure IGF-I into hypophysectomized rats caused dose-dependent stimulation of body weight, tibial epiphysial width and

thymidine incorporation into costal cartilage (Schoenle *et al.*, 1982). Recently, with a nitrogen-restricted experiment, Tomas *et al.*, (1991) demonstrated that IGF-I has a protective effect on loss of body weight during nitrogen restriction. The response is mediated by an improved nitrogen retention.

Conflicting results have been reported on actions of IGF-I in its mediating role (see review, Sara and Hall, 1990). In a study using lambs injected with bGH there was a significant effect of bGH/nutrition interaction for IGF-I but there was no such effect on body weight/components or specific GH binding to the liver (Bass *et al.*, 1991). This indicates that an increase in IGF-I does not necessarily result in increases in growth or carcass composition.

1.3.4 GH interaction in reproductive performance

GH has been found to enhance follicle-stimulating hormone-induced (FSH) differentiation of cultured rat granulosa cells. Thus, GH augments luteinising hormone receptor formation and progesterone biosynthesis (Jia *et al.*, 1986). Recently, human GH and FSH were found to have an *in vivo* synergistic stimulating effect on the ovaries of hypophysectomized rats, resulting in a greater number and a larger size of the follicles (JØrgensen & Nowak 1989).

Treatment of female rat with hGH for two weeks before mating, throughout mating and during the first seven days of pregnancy increased the number of ova ovulated. This increase resulted not only in a higher number of foetuses but also in a higher number of viable offspring (JØrgensen *et al.*, 1991). Similar results were also obtained in

cattle and gilts treated with GH. Gong *et al.* (1991) demonstrated that treatment with bGH significantly increased the population of antral follicles 2-5 mm in diameter in mature heifers. Kirkwood *et al.* (1988) reported that daily injection of GH increased ovulation rate in gilt, which is largely mediated by an elevation of local IGF-I concentrations.

GH was also shown to stimulate IGF-I secretion and enhance the effects of oestradiol and FSH on progesterone secretion from cultured porcine granulosa cells (Hsu and Hammond 1987). Substantial experimental data suggest an important role of IGF-I in granulosa cell physiology (Davoren & Hsueh, 1986; Adashi *et al.*, 1985; Davoren *et al.*, 1986). In rat granulosa cells IGF-I enhances FSH stimulated aromatase activity and progesterone biosynthesis (Davoren *et al.*, 1986; Adashi *et al.*, 1985a).

The onset of puberty was delayed in female rats receiving 1 mg rGH/day. Mean age at occurrence of vaginal patency was 37.0 days for control rats and 39.7 days for rats treated with 1 mg rGH/day (Groesbeck *et al.*, 1987). .

Additionally, it has been demonstrated previously that hGH has a lactogenic activity in rats and stimulates the proliferation of mammary gland epithelium and subsequent milk secretion (JØrgensen *et al.*, 1988).

1.4 APPLICATION OF TRANSGENIC ANIMALS

Transgenic animals are proving increasingly useful in biological studies. At least 5 areas of research can be identified as benefiting from

this technology. Firstly, in genetics where transgenesis has provided researchers with a powerful tool for studying the regulation of gene expression and effects of the foreign genes on normal development (reviewed by Gordon and Ruddle, 1985; Palmiter and Brinster, 1985; 1986; Brinster and Palmiter, 1986; Jaenisch, 1988; Gordon, 1989; Seamark, 1989; 1991; Seamark and Wells, 1993). Secondly, in immunology where transgenesis has allowed novel insights into immune mechanisms and the regulation of immune responses (see reviews, Storb *et al.*, 1986; Storb, 1987; Palmiter and Brinster, 1986; Jaenisch, 1988; Gordon, 1989). Thirdly, in pathology which has benefited from a study of the phenotype induced in transgenic mice expressing various viral and endogenous genes, thus providing valuable systems for the studies of various pathological conditions. For example, several oncogene constructs have been introduced into mice that elicit tumorigenic or other pathological changes (see reviews, Palmiter and Brinster, 1986; Gordon, 1989; Jaenisch, 1989). Fourthly, in endocrinology where numerous transgenic animals have been created with novel endocrine function providing endocrinologists with valuable insight into the regulation of endocrine systems. For example, mice transgenic with GRF (Frohman *et al.*, 1989), somatostatin (Low *et al.*, 1985) and IGF-1 (Mathews *et al.*, 1988) genes have been produced and the consequences of their expression utilized as probes, both to confirm existing and realize new features of the growth system (see reviews Brem *et al.*, 1989; Seamark, 1991).

More recently, transgenesis is beginning to realize its potential in the agriculture livestock industry. As previously indicated, the production of transgenic pigs, sheep, cattle and rabbits has been achieved. Although over 100 different genes have been transferred to

mice, the number of genes transferred to livestock animals is still limited and mostly related to transgenes encoding growth promoting hormones such as GH or growth hormone releasing factor (see recent reviews, Pursel *et al.*, 1989; 1990; 1990a; McCrane *et al.*, 1990; Rexroad *et al.*, 1990; Seamark and Wells, 1993). New transgenic breeding lines of pigs with enhanced production characteristics have been established (Vize *et al.*, 1988; Pursel *et al.*, 1989).

1.4.1 Growth regulation by expression of GH transgene

The most obvious effect of expression of rat, bovine, human, or ovine GH genes is a stimulation of growth that commences at about 3 weeks and plateaus at about 12 weeks when the mice are as much as twice their normal size (Palmiter *et al.*, 1982; 1983; Orian *et al.*, 1989). Similarly, production of excess human GRF stimulates the somatotroph cells to produce excess GH, which then elevates IGF-I mRNA and stimulates growth (Hammer *et al.*, 1985).

1.4.1.1 Constructs of GH fusion gene

Through the use of an absolute tissue-specific enhancer, the chromosomal context could be expected to restrict the level of expression of a transgene in one cell type, whereas with promoter/enhancer sequences that allow functions in many different cell types, the chromosomal influence could be expected to vary in the different cells. In attempts to gain tissue specificity and control over expression of the fusion gene in mice and other species a variety of promoter sequences have been used, which include rat Elastase-I (Ornitz *et al.*, 1985), mouse transferrin and mouse albumin (see review,

Palmiter & Brinster, 1985; Pursel *et al.*, 1990a). Recently, rat phosphoenolpyruvate carboxykinase (PEPCK) has been used as a promoter fused to the bovine GH (bGH) structural sequence. This promoter gene is specific to the liver and expression is determined on the ratio of carbohydrates to protein (McCrane *et al.*, 1988; 1990). Mouse and human metallothionein I and IIA are the most commonly used promoters fused to GH sequences. This has resulted in the fused gene being expressed in virtually all cells whilst being responsive to a multitude of inducers (glucocorticoids, metals, inflammatory signals and interferon).

1.4.1.2 Expression of GH fusion gene

Since the first expression of GH fusion gene was achieved (Palmiter *et al.*, 1982), a number of GH fusion genes, which are composed of promoter/regulator sequences from one gene fused to the structural sequences of another gene, have now been integrated into and expressed in mice, sheep and pigs.

The first GH transgenic mice were produced in 1982 by pronuclear microinjection of a DNA fragment which contained the mouse metallothionein I promoter fused to the structural gene of rGH (Palmiter *et al.*, 1982). Six of the seven animals carrying the fusion gene grew to be significantly larger than their non-transgenic littermates (Palmiter *et al.*, 1982). In the interim, human, bovine and ovine GH (oGH) genes as well as hGH-releasing factor or IGF-I genes driven by heterologous promoters, have also been introduced into the mouse genome (Palmiter *et al.*, 1983; McGrane *et al.*, 1988; Orian *et al.*, 1989; Hammer *et al.*, 1985; Mathews *et al.*, 1988). Metallothionein (MT)

promoters have been used preferentially to induce ubiquitous expression of the structural gene, thereby leading to over-production of the hormonally active protein.

The production of foreign GH in transgenic mice varies considerably from study to study, ranging from serum levels in the order of several ng/ml (Dudley & Portanova, 1987; Bartke *et al.*, 1988) to greater than 900,000 ng/ml (Palmiter *et al.*, 1983; Brem *et al.*, 1989). The ratio of MT-hGH fusion gene to MT gene expression has been shown to vary among various tissues and different transgenic mice, suggesting that the MT promoter is influenced by the integration site and tissue environment (Palmiter *et al.*, 1983; see review, Palmiter & Brinster, 1986).

To date, only microinjection of DNA into a pronucleus or nucleus has been successfully used to produce transgenic pigs and eleven different regulatory sequences fusion genes have been transferred into pigs. About 70% of the transgenic pigs that were tested expressed the integrated genes, with a range of expression from 17% to 100% (see review, Pursel *et al.*, 1990a).

The level of gene expression varied greatly among transgenic pigs that had integrated the same fusion gene. Plasma concentration at birth ranged from 3 to 949 hGH ng/ml and 5 to 944 bGH ng/ml in MT-hGH and MT-bGH transgenic pigs, respectively (Hammer *et al.*, 1985; Miller *et al.*, 1989). Pigs with high levels of expression showed evidence of ill health but interestingly, at low levels of expression about 28 ng/ml pGH in pGH transgenic pig, expression of the pGH transgene was accompanied by an marked improvement in daily weight gain and body

weight without apparent adverse effect on health (Vize *et al.*, 1988; Pursel *et al.*, 1990; 1990a).

In sheep expression of inserted genes varied from a low of 0 of 4 to a high of 3 of 3, with the overall proportion 34% (see review, Rexroad *et al.*, 1990). Species homology between a promoter and the transgenic species may be beneficial for gene expression. The construct sMT-sGH9 (sheep metallothionein-sheep GH fusion gene) resulted in very high concentration of plasma GH in lambs while mouse metallothionein and transferrin promoters elevated plasma GH, but not to the same degree. No observation of elevated growth in transgenic sheep has been made yet (reviewed by Rexroad *et al.*, 1990).

1.4.2 Regulation of GH transgene expression

In transgenic mice harbouring GH fusion genes with the MT promoter, the concentrations of hGH and bGH were frequently elevated more than 10-fold after zinc was added to the drinking water (Palmiter *et al.*, 1983; Hammer *et al.*, 1985a). Selden *et al.* (1989), using a mouse MT-I promoter, demonstrated that hGH fusion gene was regulated by glucocorticoids in transgenic mice. A 1.5- to 6.3-fold induction in serum hGH level was observed for every mouse tested and both the basal level and the glucocorticoid induction of fusion gene expression were heritable. In contrast, the addition of 1000-3000 p.p.m. zinc to the feed of transgenic pigs only resulted in a doubling of the bGH content of plasma, which was significantly less than observed in transgenic mice (Pursel *et al.*, 1990a). Recently, very encouraging results have been obtained in both transgenic mice and pigs by using a PEPCK gene promoter to regulate bovine growth hormone production.

Diets high in carbohydrate markedly depress PEPCK which reduces the level of bGH in the serum by more than 90%. The same animals, fed a high protein diet devoid of carbohydrate, responded dramatically with a rise of some 30-fold in serum bGH levels in a week (Wieghart *et al.*, 1988; McCrane *et al.*, 1988; 1990). It suggests that other variations on this `metabolic' theme of transgene controls will also emerge. However, inducibility of inserted genes, such as increased production of GH by mouse MT-bGH transgene in response to heavy metals, has not been demonstrated for sheep (see review, Rexrod *et al.*, 1990).

1.4.3 Reproductive performance in female GH transgenic mice

GH is believed to promote sex maturation in different species (Bartke, 1965; Ramaley *et al.*, 1980; Wilson, 1986) and GH-dependent IGF-I can stimulate somatic cells in female and male gonads (Adashi *et al.*, 1985; Lin *et al.*, 1986). However, GH excess in acromegaly is often associated with menstrual abnormalities, reduced libido, and impotence (Jadresic *et al.*, 1982). Similarly, transgenic mice with foreign GH gene expression and the resulting chronic elevation of plasma GH levels often exhibit reduced reproductive performance in females, ranging from delay in conception and modest increases in fetal or neonatal mortality to high incidence of infertility or complete sterility of one sex (Yun and Wagner, 1987; Bartke *et al.*, 1988; Naar *et al.*, 1991; Bartke *et al.*, 1992).

In the case of hGH transgenic mice, females expressing the transgene are almost invariably sterile (Hammer *et al.*, 1984; 1986; Yun and Wagner, 1987; Bartke *et al.*, 1988). Moreover, the introduction of the MT-hGH gene into GH-deficient little (*lit/lit*) mice produced normal

growth but rendered females sterile (Hammer *et al.*, 1984). Yun and Wagner (1987) found this sterility was not due to the inability of the females to produce ova or to defects in fertilizability of oocytes, as when they transplanted ovaries from transgenic MT-hGH females to normal females, the transplant recipients were fertile and could transmit the transgene to their progeny. Bartke *et al.* (1988) also confirmed that the 'sterile' transgenic females had normal ovarian function in that they exhibited cyclic changes in ovarian activity, ovulated, and produced fertilizable ova. However, they also showed that there was impaired release of prolactin after mating in hGH transgenic female mice, which resulted in altered luteal function (Bartke *et al.*, 1988), which accounted for their impaired fertility status. However, there are many details of the transgenes effect which remain unclear, as transgenic female mice expressing human placental GH variant (hGH•V) gene do reproduce, even though hGH•V, is very similar in action to hGH, being both somatotropic (Selden *et al.*, 1988) and lactogenic (Nickel *et al.*, 1990; Bartke *et al.*, 1990) in rodents.

Transgenic female mice expressing the bGH gene can be fertile, although there is high incidence of sterility in some of the animals lines (Naar *et al.*, 1991), and the reproductive performance of the transgenic females remains generally inferior to that of their normal female siblings. For example, when transgenic MT-bGH females are housed with a normal male, they require a longer interval to become pregnant than the non transgenic females, rarely mate in postpartum oestrus, and never become pregnant from postpartum mating (Naar *et al.*, 1991).

Interestingly, transgenic female mice expressing high levels of oGH in their serum showed almost normal reproductive performance

although the degree of homology between oGH and bGH is 99.5% (Dayhoff *et al.*, 1978) and other alteration such as growth rate and liver size in oGH mice were similar to those of rGH and hGH trangenic females (Orian *et al.*, 1989).

Transgenic female pigs and sheep expressing MT-hGH, bGH and oGH developed by gene transfer technology exhibited suppressed reproductive function, which were similar to those in transgenic mice (see recent reviews, Pursel *et al.*, 1989; Rexroad *et al.*, 1990). In addition, early death of the transgenic pigs and lambs prevented development of lines of transgenic farm animals that could be thoroughly evaluated for GH effects on reproductive performance and growth. Further intensive studies are needed to elucidate the effects of expression of GH genes on reproduction to find practical application for increasing growth and improving food utilization and body composition in livestock animals.

1.4.4 Transgenic rats

During the course of this study several lines of transgenic rats have been produced (Mullins *et al.*, 1990; Hochi *et al.*, 1990; Hammer *et al.*, 1990; Zajac *et al.*, 1994). The first success was achieved by microinjection of a 24 kb DNA fragment of the mouse ren-2 gene into the pronuclear of embryos derived from a cross between a CD female and KKY male (Mullin *et al.*, 1990). These transgenic rats provide a model where the transgene operated against a known genetic base for the hypertension. The second report related to the production of transgenic rats susceptible to experimentally induced inflammatory disease (Hammer *et al.*, 1990). A particular allele of the class I major

histocompatibility locus, HLA-27, is thought to be responsible for this condition in humans. Microinjection of HLA-27 and human beta microglobulin (hβ2m) gene into either Lew or F344 1-cell rat embryos produced transgenic rats expressing the fusion gene with HLA-B27-associated human disorder. This model is of particular importance because the equivalent transgenic mouse models showed physiologically normal expression of the HLA-B27 allele but failed to elicit features of the human disease. Indeed, rats are susceptible to several experimentally inducible arthritic diseases that cannot be modelled in the mouse. The other reports were for the introduction of bovine - lactalbumin (BLA) and rat PEPCK genes into rats (Hochi et al, 1992 Zajac *et al*, 1994).

1.4.4.1 Procedures of microinjection

Generally, the procedures used for microinjection in these studies were essentially the same as has been used for the mouse, with the exception of the use of an injection buffer which consisted of 10 mM Tris-HCL and 0.1 mM EDTA (Hochi *et al.*, 1990; Mullins *et al.*, 1990). The eggs were held in PBS (Hochi *et al.*, 1990; 1992) and Brinster's medium (Hammer *et al.*, 1990) prior to implantation into day 1 pseudopregnant recipients.

1.4.4.2 Transgenic efficiency

Four other groups have introduced foreign genes into the germ line successfully by using different strains of rats. In Mullins *et al.* (1990) study 8 progeny were obtained from the 37 microinjected eggs implanted, of which 5 were transgenic. Of these, 3 showed the

capability for germ line transmission. The same group subsequently introduced the human renin and human angiotensinogen genes into rats (Sander *et al.*, 1992). In the former case, of 87 CD zygotes microinjected and transferred, 5 transgenic offspring were obtained, 2 capable of germ line transmission. For the angiotensinogen gene, from 167 transferred eggs, 7 transgenics were born, of which 4 showed germ line transmission. Thus overall, these three examples gave a transgenic frequency of 13%, 6% and 4% respectively.

In the study in which rats harbouring the HLA-B27 and hβ2m genes were created (Hammer *et al.*, 1990), 7 of the LEW and 4 of the F344 rats developing from either 348 or 329 microinjected ova showed integration of the HLA-B27 and hβ2m genes, which gave a overall transgenic frequency of 2% and 1.2% respectively.

Of 461 microinjected embryos transferred to pseudopregnant recipients, 20 of 103 pups carried the BαLA gene, 15 of which showed the capability of germ line transmission, which gave a overall integration of 4.3% (Hochi *et al.*, 1992).

Recently, in the production of PEPCK transgenic rats, 3 of 41 rats born from the injected eggs contained the transgene and showed germline transmission (Zajac *et al*,1994)

1.5 SUMMARY

The last few years have witnessed an extraordinary increase in the use of transgenic animals. Each of the three methods (pronuclear microinjection, retrovirus infection and embryonic stem cell transfer) for

generating transgenic animals have been developed and refined and now constitute standard procedures. The most common approach is microinjection of DNA into pronuclear of one cell zygotes.

Since the initial experiment of GH transgenic mice of Brinster and Palmiter, a great number of transgenic animals expressing GH transgenes have been produced and with the potential of transgenesis to enhance livestock production efficiency many approaches have been made to obtain better control of the transgene expression. Although some progress has been made by including an appropriate combination of promoter/enhancer elements in the DNA construct *in vitro*, the problem of obtaining reliable control over the expression of the transgenes *in vivo* remains and further study is needed to fully understand factors determining the expression of GH fusion genes in transgenic animals.

Many of the GH transgenic mice generated have shown suppressed reproductive function in the females with the implication that this may be a significant constraint on the development of transgenic livestock. However, in other experiments using administered GH, it has been shown to have the potential to enhance female fertility. Thus, it is possible through appropriate control of transgene expression that GH expression could be used to advantage. Therefore it is of considerable interest to further define and elucidate the effects of heterologous GH gene expression on female fertility. The transgenic mouse model, because of its size, limits detailed investigations of the infertility problems and an argument is made that the development of a transgenic rat model could be useful in this regime.

Chapter 2
Materials and Methods

2.1 ANIMAL MAINTENANCE AND HANDLING

Laboratory rats, Hooded Wistar, were obtained from the Animal Resource Centre at the University of Adelaide or derived from breeding stock held at the CSIRO Animal House, Glenthorne. Animals were held for experiments at the Medical School Animal House of Adelaide University or Glenthorne quarantined rodent-proof rooms at 20°C in artificial light in a 12:12 (D:D) period. Human access was securely controlled. All rats were provided with unrestricted access to water and feed, ad libitum, a pelleted ration composed of 21% (w/w) protein, 6% fat and 73% carbohydrate which had a metabolizable energy content of 12.5 MJ/kg and contained 1.23% lysine, 0.39% methionine, 0.71% methionine plus cysteine, 0.26% tryptophan, 0.87% isoleucine, 1.54% calcium and 0.43% available phosphorus (Mouse Cubes, Milling Industries Ltd., S.A.). Pups were isolated from their mothers at 4 weeks after birth and caged with a littermate of the same sex.

2.2 VASECTOMY OF MALE RATS

2.2.1 Anaesthetic

Animals were anaesthetised with Avertin (Michalska, 1988). To anaesthetise a rat a solution (15 µl per gram of body weight) of Avertin was prepared as following and injected intraperitoneally. One gram of 2,2,2-tribromoethanol (Aldrich Chemicals Co., USA) was dissolved in 1 ml of tertiary amyl alcohol (2-methyl-2-butanol; BDH Chemicals, Poole, UK) and diluted with tap water to 50 ml. The solution was shaken vigorously and 3 ml aliquots were stored frozen at -20°C until needed.

2.2.2 Vasectomy

Adult male rats (12-14 week old) were anaesthetised and their abdomen shaved and swabbed with 0.05% (w/v) chlorohexidine (Delta West Ltd., Australia). All of incision about 1 cm was made in the skin about 1 cm anterior to the penis and a similar, but slightly smaller incision was made through the abdominal peritoneum. Testicular fat was located and drawn out with small curved forceps, exteriorising the testis, vas deferens and epididymis. The vas deferens was cut between single proximal and distal ligatures (surgical silk # 5.0) placed approximately 5 mm apart. The testis, vas deferens and epididymis were gently returned into abdominal wall and the skin sutured with surgical silk # 5.0. Approximately three weeks post surgery vasectomized males were mated with two females each. The females were sacrificed 12 hours after mating and oocytes collected and examined under a stereo-microscope. A successful vasectomy was confirmed if there was an absence of sperm and unfertilized eggs.

2.3 SUPEROVULATION OF DONORS

2.3.1 PMSG and hCG injection

Immature (4 week old) female rats were superovulated by intraperitoneal injection of 10 IU PMSG (Folligon, Intervet, Holland) followed 48 hours later by 10 IU hCG (Chorulon, Intervet, Holland). Immediately following the hCG injection each female was placed with an intact male. Matings were confirmed by vaginal plugs. Detection of a copulatory plug was considered day 0.5 of pregnancy.

2.3.2 FSH infusion

Pituitary FSH preparations (Folltropin-v, Vetrepharm Inc., London, Canada) were administered by continuous infusions with S.C.-implanted Alzet osmotic minipumps (Model 2001, Alza Corp, USA) to immature (4 week old) rats, which deliver approximately 1 µl/h of 6000 µg/ml solution. The pumps were inserted, under ether anaesthesia, on day -2, between 0800 and 0830 hour. At 1200 on day 0, i.e after infusion of the gonadotropin for approximately 52 hours, 10 IU hCG was injected to each donor.

2.4 EMBRYO COLLECTION

2.4.1 Embryo culture medium

The basal culture medium was similar to that developed for *in vitro* fertilisation of rat oocytes (Toyota and Chang, 1974), except that the BSA was replaced by 1 mg/ml polyvinylalcohol and the content of some supplements were modified (Zhang and Armstrong, 1990). Table 2.1 lists the formulation of the basal medium.

Table 2.1 Formulation of embryo culture medium*

Component	**Concentration**
Basal medium	
NaCl	94.6 mM
KCl	4.78 mM
$CaCl_2$	1.71 mM
KH_2O_4	1.19 mM
$MgSO_4$	1.19 mM

$NaHCO_3$	25.07 mM
Sodium lactate	21.58 mM
Sodium pyruvate	0.5 mM
Glucose	5.56 mM
Streptomycin sulphate	25 µg/ml
Penicillin G	25 U/ml
Polyvinylalcohol	1.0 mg/ml
Supplements	
ITS**	
Insulin	5 µg/ml
Transferrin	5 µg/ml
Selenium	5 µg/ml
Amino acids***	
L-Arginine.HCl	0.6 mM
L-Cystine	0.2 mM
L-Glutamine	2.0 mM
L-Histidine HCl.HO_2	0.2 mM
L-Isoleucine	0.4 mM
L-Leucine	0.4 mM
L-Lysine HCl	0.4 mM
L-Methionine	0.1 mM
L-Phenylalanine	0.2 mM
L-Threonine	0.4 mM
L-Tryptophan	0.05 mM
L-Tyrosine	0.2 mM
L-Valine	0.4 mM

* The osmolarity and pH (after the medium was equilibrated with 5% CO_2 in air) of the medium was checked in every experiment; the average osmolarity was 275-280 m Osmoles (basal medium plus supplements), and average pH was 7.2-7.3.

** ITS was added as a premixture (CR-ITS premix, Collaborative Research Inc., Bedford, MA).

*** Added as a premixed solution (GIBCO< Catalogue No. 320-1135AG).

2.4.2 Embryo collection

Mated females were killed by cervical dislocation, about 24 hour after hCG injection and the oviducts removed and transferred into a Petri dish containing 2 ml HEPES-HTF medium supplemented with BSA (5 mg/ml) and hyaluronidase (300 IU/ml; Sigma Co.). The oviduct ampullae were opened with fine watched-maker's forceps and 26 gauge needle and the cumulus enclosed eggs released. The dish was then placed on a warm plate at 37°C for 2-3 minute and as soon as the eggs had separated from the cumulus cells they were recovered with a fine "hand-pulled" glass pipette and washed in two changes of the embryo culture medium. The eggs containing pronuclei were identified under the stereo-microscope and placed in lots of approximately 30 in 30-40 µl microdrops of culture medium overlaid with paraffin oil. The eggs were then incubated at 37°C under 5% CO_2/5% O_2/90% N_2 (v/v).

2.5 CONSTRUCT OF THE PORCINE GROWTH HORMONE TRANSGENE

The porcine growth hormone gene construct used (MTIIa/pGH) was provided by the Department of Biochemistry, the University of Adelaide. The construction of this transgene has been described

previously (Vize *et al.*, 1989). Briefly, the human metallothionein IIa promoter (hMTIIa) was obtained as an 825 bp fragment which contained sequences from -765 to +60. This fragment was purified, subcloned into Hind III/Eco RI digested vector pUC19 and named pUCMT. The Eco RI insert of pPG-3, a plasmid containing a full length of porcine growth hormone (pGH) cDNA, was then isolated and subcloned into the Eco RI site of pUCMT to create plasmid pMTGH. This plasmid contains a unique Sma I site located within the region of the cDNA clone corresponding to the last exon of the genomic pGH gene. Plasmid pMTGH was digested with Sma I and ligated to a 1000 bp Sma I/Bam HI fragment, isolated from a porcine cosmid library by screening with pGH cDNA clone pGH-3, and was included in the construct to provide the DNA sequences required for polyadenylation of mRNA. The final plasmid, pHMPH.4, was then restricted with Hind III and Pvu I to release a 2.7 kb fragment containing the entire MTIIa/pGH genomic DNA region plus 120 bp of the *lac* Z gene of pUC19. This fragment was isolated by electrophoresis through low melting temperature agarose and purified by phenol/chloroform extraction and ethanol precipitation. The organization of the plasmid pHMPG.4 is presented in Fig. 2.1.

2.6 MICROINJECTION SYSTEM

2.6.1 Microscope

An inverted microscope (Diaphot, Nikon, Japan) fitted with DIC optics was used for DNA microinjection. The objective turret was fitted with a LWD 20x, LWD 40x, and 10x DIC objectives and a 4x bright field objective. Microphotographs were taken with a 35 mm automatic camera (Nikon) attached directly to a 35 mm camera bayonet fitting on

the microscope, using 50% of the total transmitted light. Photographs taken used 400 asa colour slide and film.

2.6.2 Micromanipulators

Two three-dimension movement micromanipulators (NT-8, Narishige Scientific pty. ltd., Japan) were attached to and each mounted on either side of the microscope. The left-hand manipulator controlled the injection pipette and the right-hand one the holding pipette. The microscope and manipulators were placed on a heavy slate slab which was rested on pieces of rubber tubing to prevent possible vibrations.

2.6.3 Holding pipette

Holding pipette was made from Leitz glass capillary tubing of 1.0 mm outside diameter (OD). The tubing was pulled in a Brown-Fleming horizontal pipette puller (Model P-77, Sutter Instrument Co., California, U.S.A.). The drawn-out tip of the pipette was touched on the filament of a Defonbrune microforge (Defonbrune, France) so that it adhered. Then the microforge was switched off and the tip of the pipette was pulled and broken to give a tip 40-50 μm OD. The tip of the pipette was then polished in the Defonbrune microforge to give a internal diameter (ID) of 3-4 μm. Two 90^{o} bends were then made in the pipette, using a microflame, to give a Z-shaped tip. The pipette were always made freshly before microinjection. To control meniscus movement in the holding pipette, a gas tight (threaded plunger) 0.5 ml Hamilton syringe (Alltech Pty. Ltd., Australia) was connected to a Leitz instrument holder containing the holding pipette by teflon tubing. The entire holding pipette system was filled with silicon oil (200 fluid/20 centistokes, Dow Corning, U.S.A.)

2.6.4 Injection pipette

The glass capillary tubings of 1.0 mm OD containing a filament fused to the inner wall were obtained from Clark Electromedical Instruments (Cat. No. GC 100 TF-15). The tubing was pulled mechanically in the Brown-Fleming pipette puller under conditions which give pipettes with the open tip of 0.2 μm OD and measuring 2 μm OD at the distance of 60 μm from the tip. The tip was then modified to a Z-shape as described for the holding pipette. The microinjection pipette was filled with the DNA solution through the blunt end by capillary action. The tip of the pipette was opened by gently brushing onto the tip of the holding pipette in the microinjection chamber as injection was carried out. Microinjection of DNA was controlled by connecting a Leitz instrument holder containing the microinjection pipette to a high pressure nitrogen cylinder via a 3-way valve gas flow switch (Michalska, 1988) using thick walled plastic tubing (Masterflex Tubing 6409-16, Norton Performance Plastics, U.S.A.)

2.6.5 Microinjection buffer

The HEPES-buffered Human Fallopian Tube fluid medium (HTF,Quinn *et al.*, 1984; 1985) was used to maintain stable pH during embryo microinjection and transfer. The buffer was prepared using fresh water purified by Millipore Reverse Osmosis and Milli-Q system. Concentrated stock solutions of various components were prepared according to Whittingham (1971) and stored at 4°C for up to three months and were used to make up fresh media every fortnight. The prepared media were stored at 4°C until required. The osmolarity of fresh media was routinely adjusted to 280 mOsm with water. The day before use the medium was supplemented with 5 mg/ml bovine serum

albumin (BSA, fraction V, Sigma Co., USA). The medium was filter-sterilized and warmed up to 37°C.

The composition of HEPES-HTF medium is presented in Table 2.2. All inorganic salts and glucose were of Analytical Grade and were obtained from Mallinckrodt Chemical Works, St. Louis, USA. Penicillin, lactic acid (grade DL-V, sodium salt, 60% v/v, aqueous solution) and sodium pyruvate (type II) were obtained from Sigma Co., USA.

Table 2.2 Composition of HEPES-HTF medium

•Component	Concentration (mM)
•NaCl	101.6
•KCl	4.69
•$MgSO_4 7H_2O$	0.20
•KH_2PO_4	0.37
•$CaCl_2 2H_2O$	2.04
•$NaHCO_3$	4.0
•Glucose	2.78
•SodiumPyruvate	0.33
•Sodium Lactate	21.40
•HEPES	21.00
•Penicillin	100 U/ml
•Phenol Red	0.001% (w/v)

2.6.6 Microinjection chamber

Microinjection chambers were identical to that described by Michalska (1988). A 20 mm diameter hole was drilled through the

centre of a piece of hard plastic slide (75 mmL × 24 mmW × 3-4 mmH). The hole was covered by a half of standard microscope glass slide attached to the plastic slide using bee's wax. The microscope slides were lightly siliconized (Aquasil, Pierce Chemical Co., USA; 0.2-0.5%, v/v solution) to allow the drop of medium to assume a dome shape. A 50 µl drop of HEPES-HTF medium was gently placed in a position off centre in the well and covered with sterile paraffin oil. The chambers were always freshly made and not sterilised before microinjection. Following completion of microinjection the glass bottoms were discarded and the bee's wax wiped off the plastic slides, which were kept for future use.

2.7 DNA MICROINJECTION

2.7.1 Preparation of DNA solution

The DNA samples were diluted in phosphate buffer saline-PBS, pH 7.0 (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to a concentration of 5 or 10 ng/ml, and stored frozen (-20°C) in aliquots. To prevent clogging of the injection pipettes each aliquot was centrifuged for 5 minutes at 8000 xg before use to remove any extraneous particulate matter.

2.7.2 Pronuclear microinjection

Before injection, approximately 30 eggs were taken out of culture medium and transferred into a drop of HEPES-HTF medium in the injection chamber. Embryo collection and culture before microinjection were described previously (see chapter 2.4.2). Holding and injection pipettes were inserted into right and left instrument collars respectively, using brass and rubber gaskets to ensure a tight closure. The injection

chamber was placed onto the microscope stage and the eggs brought to focus under the low magnification. Both the injection and the holding pipettes were lowered into the drop containing the eggs and brought into the same focal plane as the eggs. The microinjection was performed under 200x magnification. An egg was picked up by the holding pipette and immobilised by applying a gentle suction via the microsyringe. The egg was focused into the same focal plane as the pronucleus. Using the fine control lever of the micromanipulator, the injection pipette was moved forward. When the tip of the pipette entered the pronucleus, about 4-8 pl of the DNA solution was discharged by a short pulse of pressure (10 psi) supplied through the three way valve connected to the nitrogen cylinder. The successful microinjection was confirmed by a expansion of the pronucleus to nearly double its size. When the group of eggs was injected, the injection chamber was taken from the microscope. The eggs were placed in the microdrops of the culture medium. The next group of 30 eggs was placed into the chamber and injected. The microinjection was performed at room temperature. When all eggs had been injected, they were examined under the dissecting microscope and surviving eggs transferred into the oviducts of pseudopregnant recipient females

2.8 EMBRYO TRANSFER

2.8.1 Preparation of recipients

12-16 week old mature, virgin female Hooded Wistar rats were placed with vasectomized males on the night before microinjection. The females were checked for copulatory plugs next morning and mated ones used as embryo recipients.

2.8.2 Embryo transfer

Microinjected eggs were recovered from culture medium and placed in drops of HEPES-HTF overlaid with paraffin oil.

Recipients were anaesthetised by I.P injection of Avertin (for detail see chapters 2.2) and the lateral-abdominal hair shaven, and the skin sterilised with 0.05% (w/v) solution of chlorohexidine. One ovary was carefully drawn out through a small flank incision, holding only the surrounding adipose tissue to avoid contact with the ovary or the duct. With the aid of a microscope at 15x magnification the ovarian bursa was gently peeled with fine forceps to expose the oviductal infundibulum. Between 12 to 20 embryos were transferred to the oviduct through the infundibulum using a finely drawn glass pipette and the ovary was replaced in the abdomen. The procedure was repeated with the other oviduct. The muscle incision was sutured with silk and the skin wound was closed with skin clips (Autoclip, Mik Ron Precision, Inc., USA). The wound was sterilised with 0.05% solution of chlorohexidine.

2.8.3 Assessment of implantation rate

When each litter was weaned, the recipients were sacrificed, their reproductive tracts dissected and uteri checked for the number of implantation scars.

2.9 IDENTIFICATION OF TRANSGENEIC ANIMALS

2.9.1 Isolation of DNA

Tail tissue samples (about 1 cm) were taken from rats approximately four weeks of age and frozen at –80^{o}C until required.

The tissue sample was sliced into small sections and resuspended in a solution (1 ml) containing 1% (w/v) SDS, 50 mM Tris- HCl pH 7.5, 10 mM EDTA and 50 µg/ml proteinase K. The samples were then digested overnight at 37°C before being spun briefly at 2000 rpm to pellet any cell debri. The supernatant was then extracted twice with phenol/chloroform and once with chloroform before being digested with RNase A (50 µg/ml) for 60 minutes at 37°C. Subsequently, the samples were subjected to a single phenol/chloroform extraction followed by an additional chloroform only extraction. The DNA was then collected by ethanol precipitation and its concentration determined by measuring the UV absorbance at a wavelength of 260 nm on a Shimadzu UV-160A spectrophotometer. The integrity and contamination with RNA was then checked by running an aliquot of each preparation on an agarose mini-gel. The relationship of one A260 unit equal to 50 µg/ml was used in the calculation of DNA concentrations.

2.9.2 DNA Dot-Blot

Integration of the transgene was determined by DNA dot blot hybridization (slot and dot-blot analyses). DNA samples (approximately 5 µg) were heat denatured in 0.1 M NaOH, 2 M NaCl and filtered onto Schleicher and Schluell Nytran membrane using a Dot-Blot manifold apparatus (Millipore).

Samples were washed through with 2 M NaCl and the DNA crosslinked to the membrane using a UV Crosslinker (Stratagene). The filter was then prehybridised overnight at 42°C in a solution containing 50% Formamide, 10x Denhardts soln., 1% SDS, 1M NaCL, 50 mM Tris-HCl pH 7.5, 10% Dextran sulphate and 100 µg/ml sonicated/denatured salmon sperm DNA (Sigma). Blots were hybridised in the same solution

with a heat denatured ^{32}P labelled probe. The hybridisation probe used for analysis was the 540 bp Hind III-Ava I fragment from the 5' end of the MTIIa promoter. This probe has been successfully used to identify mice and pigs carring the MTIIa/pGH transgene (Vize *et al.*, 1988). All probes were generated using a Bresatec Hexaprime labelling kit (Bresatec, Adelaide, SA). Filters were typically washed after overnight hybridisation, at room temperature in a solution containing 2x SSC, 0.1% SDS (30 min.) followed by a wash at 42°C (30 min.). Finally the filters were washed at room temperature and 42°C in a solution containing 0.1x SSC, 0.1% SDS, before autoradiography at -80°C.

2.9.3 Polymerase chain reaction (PCR)

Transgenic animals were identified using PCR which was performed using a Bresatec Taq polymerase kit according to instructions. The two primers used were designed to amplify up a 130 bp portion of the hMTIIa promoter (primers 1367 and 1384). The master mix contained **n**x (5 µl) 10x Taq reaction buffer, **n**x (4 µl) 25 mM $MgCl_2$, **n**x (0.5 µl) 20 mM dNTP (all four), **n**x primer 1, primer 2 (between 300 500 µg/ml and **n**x (1 µl) 0.1 U/µl Taq polymerase where **n** is the number of reactions to be carried out. Aliquots (14 µl) of the master mix were then removed to each reaction tube and the volume in each tube made up to 48 µl with sterile water. Finally, template DNA (2 µl) was added to each reaction tube and the reactions sealed with the addition of sterile paraffin oil. PCR was performed with a Perkin Elmer Cetus Thermal Cycler (Norwalk, CT, USA). Each PCR run included a contamination control lacking template DNA and a tube in which a negative control DNA template was used. All manipulations above were carried out using dedicated positive displacement pipettes in a laminar flow hood to prevent contamination.

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

2.10 IDENTIFICATION OF EXPRESSION SITE

2.10.1 Isolation of RNA

Total RNA was extracted from animal tissue (0.5-1.5 g) and tissue culture cells using the guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (1987). Cells were first trypsinised from 75 cm^3 or 150 cm^3 flasks and the cell pellet washed three times in PBS prior to extraction with solution D.

Following RNA extraction, the absorbance values at 260 nm and 280 nm of each RNA sample were determined on a Beckman DU-50 spectrophotometer. The A260/A280 ratios of the RNA samples were consistently in the range 1.6-2.0. The relationship of one A260 unit equal to 40 µg/ml RNA was used in the calculation of RNA concentrations. Aliquots of RNA were analysed on 1% Agarose TBE gels to assess the integrity of the 18 and 28S ribosomal RNA species prior to use.

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

A 150 bp antisense RNA probe, containing sequence corresponding to approximately 120 bp base pairs of the 3' end of the pGH gene, was generated by T7 RNA transcription using a Bresatec kit according to the manufacturers instructions. Ten µg of the DNA vector (pSP72) containing the anti-sense DNA template for RNA synthesis was

linearized with Pvu II, separated from any uncut vector by electrophoresis and the template recovered by the GenecleanTM protocol. The template DNA was purified by gel filtration on a Sepharose-6 column (Pharmacia). One µg of this DNA was added to a reaction mix containing 0.01 M DTT, 0.04 M Tris-HCl pH 7.6, $MgCl_2$, 500 µM of each rATP, rCTP and rGTP, 50 µM rUTP, 1 µg BSA, 100 µ Ci [a-^{32}P] rUTP and 4 units of T7 RNA polymerase, in a total volume of 20 µl. The reaction was incubated at 37°C for 60 min., before the addition of DNase I (5-10 U) and further incubation at this temperature for 20 min.. The mixture was then extracted with phenol/chloroform and the probe precipitated in the presence of 0.3M Na acetate pH 5.5, tRNA (1 µg) and 3 volumes of ethanol at -80°C for 15 min and collected by centrifugation at 10,000 xg for 15 min.. The extracted probe was electrophoresed on a 6% polyacrylamide gel and located by autoradiography for 2-3 min. The gel band corresponding to the full length probe was excised and eluted overnight in 400 µl of elution buffer (0.5M ammonium acetate, 0.001M EDTA, and 0.1% SDS), at 37°C. The buffer containing the probe was then aspirated away from the gel slice and the RNA was stored at -20°C. The 150 bp probe protected a number of species in RNA from non-transgenic rats but did not protect the full 120 bp RNA expected in pGH expressing animals.

The assay was also performed using a mouse control β-actin probe supplied with the RPA IITM kit (Ambion Inc, Austin, Texas) labeled to a specific activity approximately 1 x 10^9 cpm/µg. 100,000 cpm of gel-purified 300 bp probe (containing 250 bp of sequence complementary to the mouse β-actin gene) was hybridized overnight at 55° in 20 µl of Soln. The abundance of pGH mRNA was normalised for the actin mRNA content.

2.10.3 Ribonuclease protection analysis

This assay was carried out using the Ambion RPA IITM kit (Ambion Inc, Austin, Texas) supplied by Bresatec Ltd., in accordance with the protocol provided. 20-50 µg of the test RNA and approximately 100,000 cpm of the single-stranded pGH or actin RNA probes were combined in an Eppendorf tube and pelleted by centrifugation following ethanol precipitation. The supernatant was removed and the RNA pellet dissolved in 20 µl of deionised formamide, 3 µl water and 3 µl of 10x hybridisation buffer (4M NaCl, 0.4M PIPES pH 6.4, and 0.01M EDTA). The mixture was heated to 85°C for several minutes and then incubated at 55°C overnight.

Following hybridisation, 200 µl of RNase digestion solution was added (0.3M NaCl, 0.01M Tris-HCl pH 7.5, 0.005M EDTA) with a mixture of RNase A (50 units/ml) and RNase T1 (10,000 units/ml) diluted 1/100. Each assay included a tube in which digestion buffer was added without enzyme. The reaction was incubated at 37°C for 30 min. before 300 µl of RNase inactivation precipitation mixture was added and the tubes incubated at -20°C for 15 min, and then centrifuged at 10,000 rpm for 15 min. to precipitate the hybrids. 225 µl of Solution D (see Chomczynski and Sacchi, 1987) was added, the tubes vortexed briefly and 5 µl of tRNA (1 µg/µl) added before the mixture was precipitated with an equal volume (approximately 450 µl) of isopropanol. The protected RNA was dissolved in formamide loading buffer (Maniatis *et al.*, 1982) denatured by heating at 85°C for 2 min. and analysed by electrophoresis on a 6% polyacrylamide/urea gel. The gels were vacuum dried and autoradiographed at -80°C for 24 hours (for actin) or 5 days (for pGH) using Kodak X-Omat film and photographed. For

quantitation of protected fragments, gels were analysed using a phosphoimaging system (Molecular Dynamics, Sunnyvale, California)

2.11 ASSAY OF SERUM HORMONES

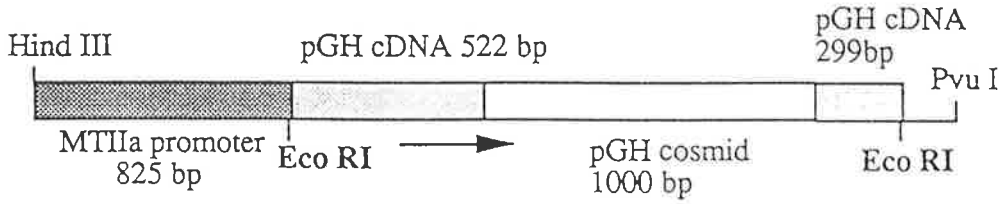
2.11.1 Growth hormone immunoassay

This assay was carried out on plasma collected from tail veins of all pups at weaning. Porcine GH in plasma was measured by a radiommunoassay using a rabbit antiserum to porcine GH (UCB-Bioproducts, Belgium; Cat No. i571). Recombinant porcine GH (Bresatec, Adelaide) was used as assay standard and for preparation of radioiodinated ligand (50 Ci/g with chloramine-T). Triplicates of 20 µl rat plasma and porcine GH standard were incubated with antiserum (final dilution 1/20000) and [^{125}I]-iodo-pGH (30000 cpm) in 0.3 ml of 50 mmol/l sodium phosphate pH 7.4 containing 5 g/l BSA for 16-20 hours at room temperature. Rabbit anti-pGH was precipitated after 30 min. incubation at room temperature following addition of 0.1 ml Cellulose-coated donkey anti-rabbit immunoglobulins (Sac-Cell, Unsworth Hall, Washington), dilution with 0.5 ml assay buffer and centrifugation. The supernatant was aspirated and the radioactivity in the pellet was measured in a gamma scintillation spectrometer.

In each assay a series of controls were included to ensure the assay was valid and to enable construction of a standard curve for determining the concentration of pGH in the plasma samples. A replicate of 5 blank tubes were set up in which the anti-pGH antibody was omitted. Radioactivity in the pellets from these tubes represents non-specific binding of the tracer to the tube and was subtracted from all the values. A second set of replicate of 5 tubes included in each

assay contained everything apart from pGH. These reference tubes determine the amount of tracer binding to the antibody in the absence of competing ligand. A final series of triplicate tubes contained dilutions of pGH (0.012-12 ng/tube) and enabled a standard curve to be derived for each assay. Both normal and transgenic rat plasma were included in the assay to act as negative and positive controls respectively.

Figure 2.1 Structure of the 2.7 kb MTIIa/pGH transgene



Chapter 3
Production of MTIIa/pGH transgenic rats

3.1 INTRODUCTION

As discussed in the literature review, the potential of GH-transgenic animals in the study of growth regulation has yet to be fully realised. The availability of GH-transgenic livestock is still limited, and the small size of mice limits the volume of blood that can be collected upon repeated sampling, which constrains experimental design in physiological and pharmacological investigations. Very sensitive assay techniques are also required to allow reliable assay of growth factors in blood without traumatising or sacrificing transgenic mice. Another limitation with mice has been the continuing problem of regulating transgene expression. With most strains of GH-transgenic mice available the foreign GH protein is produced constitutively, and the continuous expression of GH can cause the problem of pathology often observed (Orian, *et al.*, 1988; Doi, *et al.*, 1988; Brem, *et al.*, 1989; Quaife, *et al.*, 1989), although recently, Murray *et al.* (1989) have obtained more controlled regulation using the transgene employing the sheep metallothionein regulator.

Rats have proven to be useful and convenient laboratory animals for research into growth regulation and many recent developments in understanding GH action and growth regulation are derived from studies of rats that are GH-deficient, such as genetically dwarf (reviewed by Frasier, 1983) and surgically hypophysectomized rats (Bates, *et al.* 1964; Emerson, 1973). Much less common however, are studies of GH actions in normal intact rats (Groesbeck, *et al.*, 1987). The aim of this study was to establish a strain of transgenic rats containing an exogenous GH transgene whose expression could be modulated remotely through dietary manipulation and its effects observed without the stress of handling and GH injection.

At the time of initiating the study, no transgenic rats had been produced. However, a novel superovulation regime had recently been described which resulted in high yields of eggs and this was adapted to the present study, and comparison made with simple regimes.

3.2 EXPERIMENTAL

Animals holding facilities and procedures were performed at two animal house, Glenthorne Animal House, the CSIRO animal headquarter and Adelaide University Animal House in Medical School. The study was approved by the Biohazards Committee of the University of Adelaide in consultation with the Genetic Manipulation Advisory Committee of the Commonwealth of Australia, and the Animal Experimental Ethics Committees of the CSIRO Division of Human Nutrition and the University of Adelaide.

3.2.1 Microinjection procedures

Procedures for microinjection followed the protocols set out in Chapter 2.6 and 2.7. Pronuclear microinjections were carried out at 2 o'clock in the afternoon, 26 hours after hCG injection. Embryos surviving microinjection were transferred into the oviducts of pseudopregnant recipients (see Chapter 2.8 for the details).

3.2.2 Monitoring of recipients

Surgical procedures were carried out following protocols outlined in Chapter 2.8. Following embryo transfer, recipients were maintained separately to term. The number of pups was recorded at birth and all recipients were sacrificed when the baby rats were weaned.

3.2.3 Assessment of DNA integration

Transgenic animals were identified from tissue samples taken at four to five week of age (see Chapter 2.9 for details). Transgenic founder animals (F_0, rats born from injected embryos) were identified by DNA dot-blot. Transgenics in subsequent generations (F_1, F_2.... etc) were determined by either DNA dot-blot or PCR techniques (see Chapter 2.9 for details).

3.2.4 Assessment of transgene expression

Porcine GH radioimmunoassay was carried out on plasma collected from tail veins of all pups at weaning (see Chapter 2.11 for details). Rats with levels of porcine GH higher than negative controls were considered as transgenic animals expressing the transgene.

3.2.5 Development of superovulation

Comparison was made between the procedure used in the laboratory based on PMSG and the efficacy of the novel procedure based on continuous infusion of FSH (Armstrong, 1988) using Alzet minipump, as follow:

In the PMSG procedure (Treatment 1): 4 week old female Hooded Wistar rats were injected intraperitoneally with 10 IU PMSG followed 48 hours later by 10 IU hCG.

In the FSH infusion procedure (Treatment 2): pituitary FSH (6000 µg/ml) was administered by continuous infusions with S.C.-implanted Alzet osmotic minipumps to 4 week old female rats at the rate of 1 µl 6000 µg/ml/h solution. The pumps were implanted subcutaneously under ether anaesthesia as described by the

manipulation on day -2 between 8 to 8:30 in the morning. After infusion of the gonadotropin for approximately 52 hours, at noon 12 on day 0, 10 IU hCG was injected to each donor (see Chapter 2.3 for details).

The young female rats were paired with intact fertile males when hCG was injected. 24 hours following hCG injection zygotes were recovered by opening ampullae of the oviducts (see details in Chapter 2.4).

3.2.6 Microinjection experiment

Rat zygotes were microinjected with the human metallothionein II promoter/porcine growth hormone fusion gene; see Chapter 2.5 for details. 4 to 8 pls of 5 ng/μl and 10 ng/μl of DNA solution were injected into embryos to select suitable DNA concentration for rat embryo microinjection.

3.3 STATISTICAL ANALYSIS

Statistical differences between the two groups in the superovulation experiment were tested by Student's *t*-test. The differences between the two groups in microinjection of two concentrations of DNA solution were tested by x^2-test.

3.4 RESULTS

3.4.1 Superovulation

Table 3.1 provides a summary of results of applying the 2 superovulation procedures in immature rats. The average number of

oocytes recovered in rats treated with continuous infusion of FSH was 56 (ranging from 38 to 84) which was twice that obtained from the PMSG and hCG treated animals ($p<0.001$). The mean number of fertilised eggs in FSH-treated group was 45, which was significantly greater than 18, the mean number of fertilised eggs recovered in PMSG-treated group. However, rates of fertilisation in both groups were not statistically different from each other, which were 81 per cent in FSH fusion group and 75 per cent in PMSG treated group respectively.

3.4.2 Integration frequency

The effect of DNA concentration on integration and expression frequency is shown in table 3.2. These results indicate that microinjection of different amounts of DNA did not alter the number of pups born per procedure when embryos were transferred to young females at 30 per recipient, with a overall efficiency of producing live animals from injected and transferred eggs of approximately 11.6% and 9.5% in the two groups respectively. However, the frequency of transgene integration increased from 13% to 60% when the concentration of DNA injected into embryo was reduced from 10 to 5 ng/μl. The overall efficiency of the transgene integration was 5.8% in the 5 ng/μl dose group, which was significantly greater than 1.5% obtained in the 10 ng/μl group ($p<0.01$). The expression of integrated transgene at the lower microinjection dose was 17%. None of the animals produced at the higher dose were found expressing the transgene.

3.4.3 Production of hMTIIa/pGH transgenic rats

As is summarized in table 3.3, 748 embryos were microinjected with the concentration of 5 ng/μl hMTIIa/pGH DNA solution causing

lysis in 27% of cases. 546 intact injected embryos were transferred to 17 recipients resulting in the birth of 70 pups to 14 foster dams. Thus 13% of injected embryos implanted into pseudopregnant hosts survived in *utero* (table 3.3). Between 1 to 130 copies of the hMTIIa/pGH transgene per nucleus were detected by dot blot of rat tail DNA from 39 pups which gave a DNA integration frequency of 56%, and an overall efficiency of producing transgenics from the injected and transferred embryos of approximately 7% (table 3.3).

3.4.4 Expression of hMTIIa/pGH in transgenic rats

Eleven (28%) of the F0 transgenic rats expressed the foreign gene at weaning as determined by assay of pGH protein in their blood. No immunoreactive pGH was detectable (<7 ng/ml) in plasma from any of non-transgenic littermates nor in the other 28 F0 pups whose DNA contained the transgene. Levels of pGH protein in plasma from the F0 rats which expressed the transgene varied from 18 to 1190 ng/ml (table 3.4). No relationship was found between the number of copies of the transgene incorporated and the amount of pGH protein in blood at weaning. As is shown by the data printed in table 3.4, nine of the 11 expressing primary transgenic rats grew at least 24% faster than normal from weaning to 12 weeks of age. The mean plasma pGH at 4 weeks of age was >42 ng/ml in these faster-growing F0 transgenic rats. Two F0 transgenic female rats (D and M 3.4) produced pGH in their plasma at low concentrations (18 and 23 ng/ml) and grew at normal rates.

3.5 DISCUSSION

The pituitary gonadotrophic hormones, FSH and LH, are known to play separate but complementary roles in the processes of follicular growth and maturation that lead to ovulation. The gonadotropin most widely used for induction of ovulation in laboratory rodents is the equine chorionic gonadotropin, PMSG, which possesses both FSH and LH activities inherent in the same protein molecule (Papkoff, 1974). While this gonadotropin is highly effective in controlling time of ovulation and inducing mild degrees of superovulation, difficulties have been encountered in the rat where attempts have been made to utilize it to increase the ovulation rate. The problems reported include poor control of time of ovulation (Walton *et al.*, 1983), decreased rates of fertilization *in vivo* and *in vitro* (Austin, 1950; Evans and Armstrong, 1984), abnormalities in embryo development (Miller and Armstrong, 1981; Sherman *et al.*, 1982), and failure of implantation (Miller and Armstrong, 1981a).

The extent to which these difficulties relate to the long half-life of PMSG in the circulation, as has been demonstrated in both laboratory (Sasamoto *et al.*, 1972; Walton and Armstrong, 1981) and domestic farm animals (Bindon and Piper, 1977), is uncertain.

As previously reported by Armstrong and Opavsky (1988) in Sprague-Dawley rats, superovulation rates could be improved by continuous infusion of an FSH preparation and this has been confirmed in the present studies with Hooded Wistar rats. Because of the high cost of the minipumps and commercial preparations of FSH available, these improved procedures could not be used routinely, but it remained

the method of choice when large numbers of fertilized eggs were required for specific experiments.

Brinster *et al.*, (1985) demonstrated that injection of large amounts of DNA into mouse eggs was toxic, as measured by a decrease in the number of eggs that developed to the morula/blastocyst stage in culture and a lower recovery of fetuses after transfer of injected eggs to foster mothers. The overall efficiency of obtaining fetuses that had integrated the foreign DNA was best when the DNA concentration was 1 ng/µl and 2 to 3 pl of DNA solution with 600 to 1000 copies of the transgene were injected into pronuclear of the zygotes. However, according to the results obtained in this laboratory, a reasonably high recovery of fetuses and integration frequency were obtained when a concentration of 5 ng/µl of this gene construct was injected to mouse zygotes and 10 ng/µl to pig embryos (French, 1993). Comparison of 5 ng and 10 ng/µl DNA solution indicated in rats similar recovery of newborn animals in both groups following transfer at a rate of 30 zygotes to each foster mother. However, significantly higher rates of integration and expression of the transgene were achieved when the lower concentration of DNA solution were injected to embryos. Regardless of any other effects, the concentration of 5 ng/µl DNA proved suitable for microinjection into single cell rat embryos, which was utilized in all subsequent work aimed at the production of hMTIIa/pGH transgenic rats.

As indicated previously (Walton *et al.*, 1987), one of the major obstacles to successful gene transfer is the lysis of a large number of eggs following penetration of the injection pipette into zygote membrane. Walton *et al.* (1987) observed that the use of large pipettes (0.7 µm OD

at the tip and 7.5 μm OD across the shaft 80 μm from the tip) resulted in lysis of 30% of mouse eggs within one hour after microinjection. Brinster *et al.* (1985) demonstrated that the frequency of integration of foreign DNA is affected by buffer composition, conformation of the DNA and the skill and experience of the microinjector. In this study modified techniques of microinjection were used , which included usage of extremely sharp injection pipette (0.2 μm OD at the tip), critical timing of pronuclear injection and careful introduction of injection pipette into pronucleus of the zygotes, to minimize damaging the plasma membrane.

During the course of the present study, several groups have reported transgenic rats produced by microinjection of different DNA transgenes into rat zygotes (Hochi *et al.*, 1990; Mullins *et al.*, 1990; Hammer *et al.*, 1990). However, only one group has described their procedures in detail. Hochi *et al.* (1990, 1992) observed that the plasma membranes of rat eggs were readily damaged by penetration of the pipette and only 40 per cent of injected eggs survived microinjection, a much smaller percentage than obtained in the present studies. Comparison of the procedures used in the two studies revealed three critical technical differences. Firstly, the injection pipettes used in this study were made with a mechanical puller and used for microinjection without further treatment. In contrast, Hochi *et al.* subjected their pipettes to extensive treatment including polishing with a grinder, washing with sulphuric acid and hydrofluoric acid and coating with SIGMACOAT. Secondly, the studies differed in the timing of embryo collection and microinjection. In this study the zygotes were collected and injected 8 hours earlier than in the study of Hochi *et al.*. Thirdly, in this study injected embryos were transferred into the oviducts of synchronized pseudopregnant recipients immediately following

microinjection rather than after overnight culture as in Hochi's study. These might be sufficient to the reason for the difference of survival rate of embryo following microinjection.

The result of 56 per cent integration obtained in this study is comparable to 62.5 per cent integration in the other transgenic rat study using a mouse Ren-2 transgene reported by Mullins *et al.* (1990) but higher than an integration rate of 30 per cent obtained in mice in this laboratory some years ago in studies using the same transgene construct (Michalska, 1988) or by other researchers using other MT GH transgenes (Hammer *et al.*, 1985; Brinster *et al.*, 1985). Hammer *et al.* (1990) and Hochi *et al.* (1992) reported that the ratio of integration of the fusion genes in transgenic rat were 25 and 20 per cent respectively, which is comparable to that obtained in transgenic mice with the same fusion genes (Hammer *et al.*, 1989; Hochi *et al.*, 1992). More data is needed to determine whether the higher integration of foreign DNA into the rat genome reflects a peculiarity of the rat genome.

The number of copies of the MTIIa/pGH transgene inserts integrated in transgenic rats genome ranged from 1 to 130 and no correlation between expression of the transgene and the number of the copies integrated in transgenic rats observed in this study, which is consistent with the results obtained in transgenic mice with the same gene construct (Michalska, 1988).

In conclusion, with an embryo survival rate of 73 per cent following microinjection, a 56 per cent integration of hMTIIa/pGH transgene with founder rats born from injected embryos after embryo transfer and 13 per cent of the injected embryos resulting in pups, the

study demonstrates that gene transfer in rats using microinjection of the transgene to pronucleus of single cell rat zygotes can be done with a success rate equivalent to that now routinely achieved with transgenic mice.

Table 3.1 Superovulation in rats treated with FSH infusion and PMSG injection

Treatment	N	Eggs recovered	Fertilised eggs
FSH	25	56±2.8***	45±3.1*** (81%)
PMSG	25	24±1.2	18±0.6 (75%)

Values are mean ± s.e.m.

*** **P<0.001.**

Table 3.2 Effect of DNA Concentration on Integration and Expression Frequency

DNA (ng/μl)	No. embryo recipients	No. embryos transferred	No. pups born	Transgenics	No. pups expressing pGH
10	9	267	31(11.6%)	4(1.5%)	0
5	12	398	38(9.5%)	23(5.8%)**	4(1%)***

** P<0.01.

*** P<0.001.

Table 3.3 Production of hMTIIa/pGH Transgenic Rats

No. of embryos			No. of foster	No. of pups	
Collected	**Injected**	**Transferred**	**Dams**	**Born**	**Transgenic**[1]
923	748	546	17	70(13%)	39(7%)

1. Transgenic rats were defined as those whose genomic DNA contained one or more copies of at least a part of the transgene.

Table 3.4 Expression of hMTIIa/pGH in Founder (F_0) transgenic rats

Rat ID	Sex	pGH (ng/ml)	Relative growth[1]
A	M	64	1.24
C	F	800	1.81
D	F	18	1.20[2]
F	M	85	1.28
G	F	1190	2.00
H	F	1170	1.97
I	F	23	0.96
J	M	360	1.41
K	F	710	1.78
L	M	530	1.25
M	F	42	1.41

1. Relative growth rate is the weight of the transgenic rat at 12 weeks of age divided by the mean weight of non-transgenic littermates of the same sex.
2. There were no other females in the litter in which Founder D was born, and her growth rate is expressed relative to the average weight of her three non-transgenic daughters at 12 weeks of age.

Figure 3.1 Transgenic rats and their non-transgenic littermates

The size difference between transgenic and non-transgenic rats is illustrated. The photographs were taken when the rats were 12 week old.

Photograph 1

The large rat was Founder G (1190 ng/ml pGH) and the smaller one was her sex-matched non-transgenic littermate.

Photograph 2

Transgenic Founder H (1170 ng/ml pGH) and her female non-transgenic littermate

Photograph 3

Transgenic Founder K (710 ng/ml pGH) and her non-transgenic littermate

1

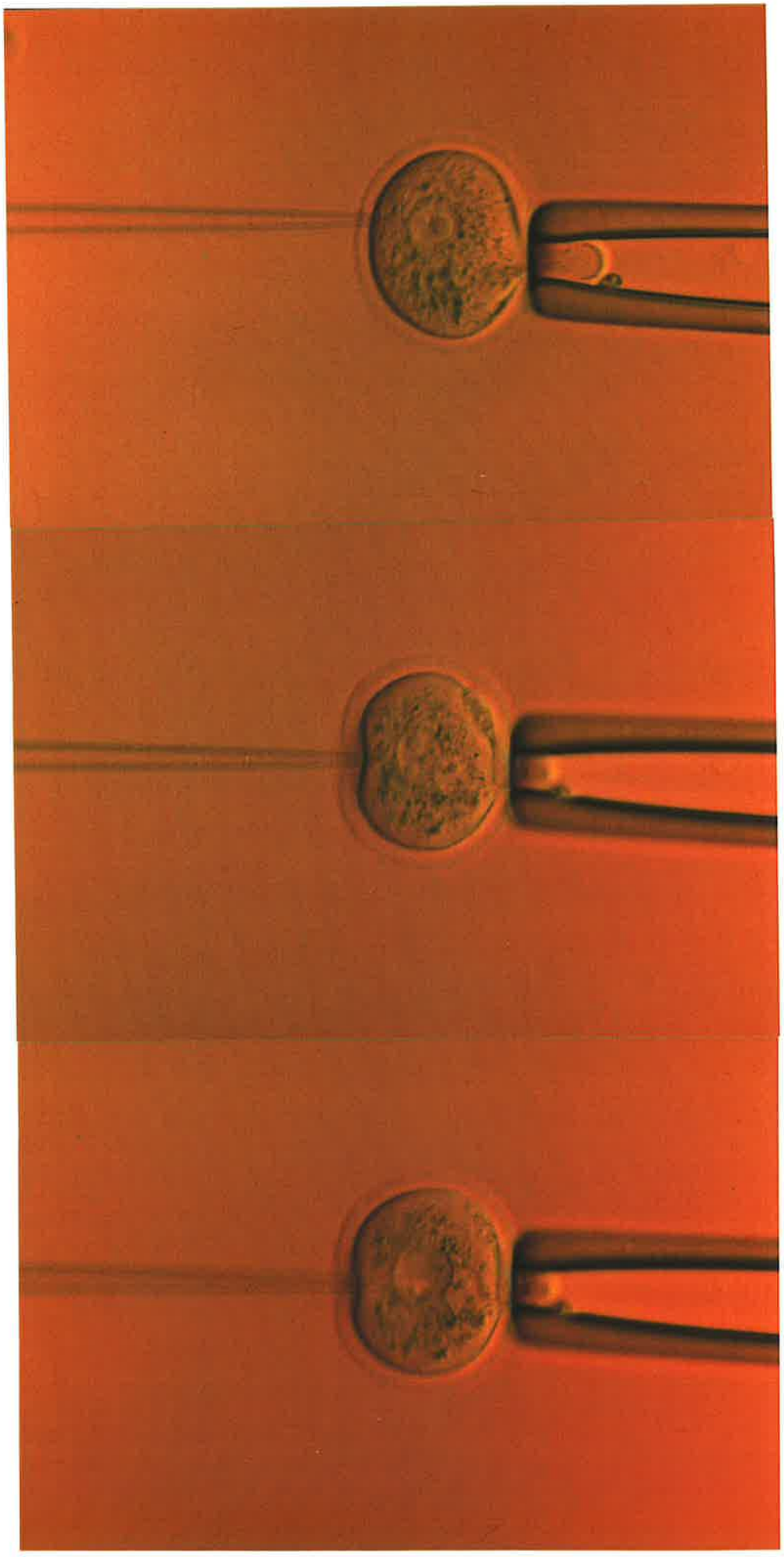
2

3

Figure 3.2 One cell rat zygotes before and after microinjection

Photograph a and b are fertilized eggs just before microinjection.

Photograph c is a zygote following pronuclear microinjection.


a
b
c

Chapter 4
Assessment of MTIIa/pGH transgenic rats

4.1 INTRODUCTION

Production of transgenic mice expressing heterologous GH genes has provided an important additional model of GH overproduction (Palmiter *et al.*, 1982; 1983; Hammer *et al.*, 1985; 1985a; Morello *et al.*, 1986). Most of the transgenic mice produced so far have responded to the extra GH by growing at an accelerated rate, eventually achieving a size almost twice that of their non-transgenic littermates. Endocrine studies of such giant mice demonstrated elevated levels of circulating GH (up to several hundred-fold greater than normal) and a 2- to 3-fold increase in the concentration of plasma IGF-1 (Palmiter *et al.*, 1982; 1983; Hammer *et al.*, 1985; Orian *et al.*, 1989). Accompanying the dramatic changes in size and body weight, the transgenic mice showed an increase in both the absolute and relative weights of their internal organs (Hammer *et al.*, 1985a; Shea *et al.*, 1987; Brem *et al.*, 1989). These changes probably reflect the lack of normal endogenous regulatory control of GH secretion seen in these animals due to GH being produced in tissues other than the pituitary (mainly liver, kidney), with the pituitary somatotroph cells themselves showing histological and biochemical evidence of regression (Palmiter *et al.*, 1983).

The utility of fusion genes in transgenic experiments is determined by the various parameters regulating the expression of the fusion promoter. In many studies, the use of strong promoters such as mouse and human metallothionein regulating elements, fused to the coding region of GH genes, has resulted in expression of the fusion genes following the pattern of the endogenous metallothionein genes with respect to both localisation and inducibility with heavy metals. However, the pattern of response is not necessarily identical to that of

the endogenous mouse (Mayo *et al.*, 1982), and human (Karin and Hershmann, 1979). To ensure better control of the transgene, additional regulatory sequences have been included or the promoter sequences modified.

This study is based on transgenic rats produced using a transgene comprised of a modified human metallothionien promoter attached to a porcine GH gene construct. Model experiments in transgenic mice demonstrated the construct performed to produce modestly elevated levels of circulating GH without basal or runaway expression (Michalska, 1988).

The investigations in this chapter were undertaken to extend the studies of transgenic mice to provide further details of the consequences of the expression of MTIIa/pGH gene construct on endocrine and metabolic state and somatic growth. An approach was also made to investigate if the expression of this transgene can be regulated in transgenic rats.

4.2 EXPERIMENTAL

4.2.1 Animal selection

From the 11 rats which were expressing the transgene, one male and three females were mated with non-transgenic rats. Blood samples and tail tissues were taken at 4 weeks of age from the offspring (F1) to determine the inheritance of integration and expression of the transgene. Offspring from animal A, C and D were located at CSIRO animal headquarter, Glenthorne Animal House, SA. The rats born from animal M were maintained in Animal Resource Centre at the University

of Adelaide. The conditions of maintenance were described previously (see chapter 2.1). The growth of both transgenic and non-transgenic littermates was recorded weekly. At 12 weeks of age the F1 transgenic female rats were mated by non-transgenic males and F1 transgenic males were mated with unrelated non-transgenic females. Further tests of inheritance of the integration and expression of the transgene and reproduction performance were carried out in F2 transgenic rats. Transgenic animals with normal fertility and stable inheritance of the transgene were selected as breeding lines.

4.2.2 Heritability of the transgene

The DNA samples of the offspring of the transgenic founder (F_0) D and M (selected as breeding lines) were analysed to determine if the foreign gene sequences were transmitted to the consequent progeny. The DNA samples of the progeny along with the samples of appropriate positive and negative controls were analysed by PCR (see chapter 2.9.4). The heritability of the expression was determined by measuring the levels of pGH in plasma which was collected from the tail veins of all pups at weaning (see chapter 2.11 for details).

4.2.3 Determination of the timing of growth response

Four litters of rats from F3 and F4 of M animal line were used for this investigation. The individual body weights of the 4 litters of rats were examined daily between 2 and 5 weeks of age. Average daily weight gain was determined on a daily basis. Non-transgenic littermates were used as controls to compare the daily gain of transgenic rats. Blood samples for GH measurement and tail tissues for PCR analysis were taken at 3 weeks of age from these rats.

4.2.4 Growth of transgenic rats

The growth of transgenic offspring from founder D and M was monitored weekly for 3 generations from 4 weeks to 12 weeks of age and compared with that of their non-transgenic, sex matched littermates. The relative weights were the ratio of body weight of transgenic rats to mean body weight of non-transgenic, sex matched littermates.

4.2.5 Determination of expression sites

Expressing sites of MTIIa/pGH transgene in these 2 transgenic animal lines were determined by Ribonuclease Protection Analysis (RPA). Two transgenic female rats from each animal line plus one non-transgenic littermate were killed and great care was taken to collect sample tissue from liver, kidney, heart, spleen, muscle, intestine, uterus and ovary. RNA for the analysis was extracted from these tissues. Antisense RNA probes were prepared using a Bresatec *in vitro* RNA transcription kit according to the manufacturers instructions. RPA was carried out using the Ambion RPA IITM kit supplied by Bresatec Ltd., in accordance with the protocol provided (see chapter 2.10 for all the details).

4.2.6 Regulation of transgene expression

Experiment 1

To investigate the induction of GH expression in transgenic rats, zinc sulphate diluted in distilled water at a concentration of 25 mM, as daily drinking water, was given to one litter (including transgenics and non-transgenic littermates) of the F1 rats of animal D, starting at 14 weeks of age for a two week period. Non-transgenic littermates were used as controls. Blood samples from these rats were taken prior to

zinc water treatment (at 14 weeks of age), at the last day of the treatment (at 16 weeks of age), and on the day two weeks after zinc water was removed (at 18 weeks of age). Animals showing increased levels of circulating GH were selected as breeding stud for further regulation studies. The same experiment was also carried out with one litter of F1 rats derived from founder M.

Experiment 2

To determine the most suitable concentration of Zn^{++}, 30 transgenic female rats and 30 non-transgenic littermates from the fourth generation of D animal line were used for this study. The rats were randomly divided into 5 groups with 6 rats in each. All transgenic groups had one matched non-transgenic group as a control. Zinc water at the concentration of 5, 10, 15 and 20 mM was given to each of 4 groups, with another group of rats remaining on normal drinking water. At 8 weeks of age the rats were placed into the experimental cages with 3 or 4 in each on normal drinking water for acclimatisation. And at 9 weeks of age the normal drinking water was replaced by the zinc water for a period of 3 weeks. At 12 weeks of age the zinc water was removed, the rats were transferred back to the big cages with normal drinking water on. Blood samples for GH measurement were taken from the rats at 8, 12 (on the last day with zinc water on) and 16 weeks of age. Body weights of these rats were examined on weekly bases from 4 weeks to 16 weeks of age.

Experiment 3

To examine the effect of induced GH expression on growth, 24 transgenic female rats and 12 non-transgenic female littermates from the fourth generation of animal D were used for this investigation. To

enhance GH expression 12 of the transgenic and 6 non-transgenic female rats were exposed to water containing 10 mM zinc sulphate for 3 weeks starting at 9 weeks of age whilst the remaining 12 transgenic and 6 non-transgenic rats remained on normal drinking water. At 12 weeks of age 6 transgenic and 6 non-transgenic rats on zinc water, and 6 transgenic rats on normal drinking water were killed by CO_2. The remaining 18 rats were killed at 30 weeks of age.

Body weight of transgenic rats and non-transgenic littermates was recorded on weekly bases. In order to investigate the effects of porcine GH gene expression on the internal organs, autopsy was performed on all the rats in this experiment and the weights of liver, kidney, heart, spleen and lung were determined individually immediately after the rats were killed.

4.2.7 Effect of constitutive pGH expression on growth of internal organs

To investigate the effect of constitutive pGH expression on the growth of internal organs, 12 transgenic and 12 non-transgenic littermates in M animal line were used. Six transgenic female rats and six non-transgenic sex-matched littermates were killed at 12 weeks of age and remaining rats killed at 30 weeks of age. The body weight and the weights of liver, kidney, heart, spleen and lung were recorded and the ratios of organ weight in relation to body weight determined.

4.3 STATISTICAL ANALYSIS

All the data are presented as means $\pm$ s.e.m.. Statistical differences between the two groups, transgenic rats and non-transgenic

controls, in absolute and relative growth and levels of pGH in plasma were tested by Student's *t*-test. The differences between the three groups, transgenic zinc-treated , transgenic and non-transgenic zinc-treated controls in effect of zinc on growth were compared by ANOVA (SAS Institute Inc., 1988). Comparisons with $p>0.05$ were not considered significant.

4.4 RESULTS

4.4.1 Selection of animals

To assess stability of the transgene integration and expression, heritability of transgenic genotype and phenotype were examined. Three fast growing F0 transgenic rats (A, C and M) were mated with unrelated non-transgenic animals. Of 19 pups from 2 litters derived from founder A (male) 5 inherited the transgene with the same number of copies per cell as their father, however, at weaning none of the pups spontaneously produced porcine GH in their plasma (<8 ng/ml) and all grew normally. This contrasts with their transgenic father who grew 24% faster than normal and whose plasma contained 64 ng/ml pGH at weaning (Figure 4.1 a).

Founder C produced a litter of 7 male and 6 female pups. At weaning only one female of the litter (C1.13) was identified to be transgenic by PCR and she had plasma levels of pGH (720 ng/ml), similar to her mother at the same age (800 ng/ml). Like her mother, whose growth rate was 81% greater than her female littermates, C1.13 grew 72% faster than her sex-matched non-transgenic sisters. The concentrations of pGH in plasma from the other 12 pups in this litter were <25 ng/ml and all grew at normal rates. One litter of 6 male and 7

female pups derived from C1.13 did not produce any pGH and grew normally (figure 4.1.b).

Female founder D (130 copies of the transgene per nucleus) had low but detectable concentration of pGH in plasma collected at weaning. This transgenic female produced a litter of 7 male and 5 female pups. Six of the pups (50%) inherited the transgene and their DNA had the same number of copies of the foreign gene as their mother. Like their maternal transgenic founder, whose plasma at weaning contained 18 ng/ml pGH, F1 transgenic rats of this genotype had between 13 and 22 ng/ml pGH in their plasma at weaning. None of these low level expressing F1 transgenic rats grew faster than their non-transgenic sex-matched siblings. The F1 male D1.6 was mated with three non-transgenic females to produce a large number of transgenic rats for further breeding.

Founder M (female, 1 to 2 transgene copies per cell) produced a litter of 2 male and 6 female pups. One daughter (M1.4) had pGH levels at 25 ng/ml at weaning, which was slightly lower than that of her mother at the same age (42 ng/ml). M1.4 grew faster (+64%) than her non-transgenic siblings as had her mother (+41%). From the first litter of 3 males and 3 females produced by transgenic rat M1.4, the concentrations of porcine GH in plasma of the three males were 49, 46 and 37 ng/ml. Although they had no comparable sex-matched littermates the 3 male rats grew at a greater than normal rate. One female (M1.4.6) produced pGH at the level of 68 ng/ml in plasma and grew 51% faster than her non-transgenic female littermates. A second litter of 6 males and 5 females from M1.4 included one male (M1.4.11) which had a level of 42 ng/ml pGH in his plasma at 4 weeks of age and

grew 27% greater than his non-transgenic male littermates at 12 weeks of age. One female (M1.4.16) produced 32 ng/ml pGH at the same age and grew 45% greater than her non-transgenic female littermates, which was similar to the growth of the maternal transgenic founder M. Transgenic male M1.4.11 was mated with 7 unrelated non-transgenic females to produce a number of transgenic female rats for further breeding and fertility studies.

4.4.2 Inheritance of the transgene integration and expression

4.4.2.1 Transmission of the fusion gene in D animal line

The inheritance of the transgene is shown in the pedigree tree for D line (figure 4.1 c). PCR analysis indicated that 15 of 27 rats (56%), in three litters born from non-transgenic females mated by D1.6, contained the transgene in their DNA (figure 4.3) and had plasma levels of pGH ranging from 12 to 24 ng/ml at weaning. To minimise the housing requirement for the large number of transgenic female rats required for subsequent fertility studies (see chapter 5 for details), only female transgenic rats were used for breeding from the third generation and new born females analysed by PCR and GH RIA to identify transgenics. Investigation for a further 3 generations revealed 103 of 206 female (50%) rats born from transgenic females mated by unrelated non-transgenic males as transgenic and all expressed porcine GH protein, resulting in serum levels ranging from 13 to 29 ng/ml, which was similar to that the maternal transgenic founder D (data not shown).

4.4.2.2 Transmission of the fusion gene in M animal line

The transmission of the fusion gene in M genotype is summarised in pedigree tree (figure 4.2). 26 of 51 (51%) F3 rats born from the 7 non-transgenic females mated by M1.4.11 were positive to PCR (figure 4.4) and they all produced pGH protein in plasma at weaning at levels ranging from 35 to 65 ng/ml. Continuous investigation of this inserted gene transmission for 7 consequent generations indicated that the fusion gene in this animal line was stably inherited with 41 of 80 (51%) rats born from transgenic females mated by unrelated non-transgenic males being positive by PCR with serum pGH levels ranging in concentration from 32 to 60 ng/ml (Figure 4.2).

4.4.3 Timing of growth response to porcine GH

Figure 4.5 demonstrates the daily weight gain of transgenic rats vs their non-transgenic littermates (female and male) in M animal line from postpartum day 21 to 34. From 15 to 21 days of age average daily gain of transgenic female rats ranged from 1.5 grams on day 15 to 3.25 grams on day 21, which was comparable to that in non-transgenic female littermates (1.7 grams on day 15 and 3 grams on day 21, data not shown in figure 4.5). However, on day 23 transgenic females had significantly superior growth rate when compared to their non-transgenic littermates, with a average daily gain of 5.8 grams, whilst it was 3.3 grams in their non-transgenic female littermates ($P<0.001$). From 23 days of age to the end of daily monitoring period (34 days of age) all transgenic females had shown a significantly increased daily growth increment compared to the non-transgenic littermates with the only exception being on day 32 (figure 4.5).

This increase in growth rate was not as clearly evident in transgenic males of the same line (figure 4.5). During the period of investigation, the average daily gain of transgenic males ranged from 2 grams on day 21 to 8.57 grams on day 32, which was more variable than the daily gain in transgenic females. Apparently, no stably increased growth occurred in transgenic males when the daily gains were compared to that in non-transgenic male littermates, which ranged from 3.25 to 8 grams during the period of investigation (figure 4.5).

4.4.4 Growth of transgenic rats

4.4.4.1 Growth of transgenic rats in M animal line

The growth of transgenic rats was monitored weekly starting from the age of 3 weeks to the age of 12 weeks and compared with that of their non-transgenic, sex and age matched siblings. The data obtained from the rats in the third generation were used here as a example of growth rate for M animal line, which was shown in the figure 4.6 (both females and males). The average body weight of transgenic female rats at 3 weeks of age was the same as that of their non-transgenic littermates (48.4$\pm$1.6 grams vs 49.1$\pm$1.7 grams). However, at 4 weeks of age transgenic females showed a significantly increased body weight when compared to that of non-transgenic littermates (82.5$\pm$7.7 grams vs 64.9$\pm$3.5 grams, $p<0.05$). As the animals aged, the differences of mean body weights between transgenic females and their non-transgenic littermates became more pronounced. At 12 weeks of age transgenic females had average body weight of 323.3 $\pm$ 4.7 grams, which was 42% increased compared to 228.1 $\pm$ 8.6 grams of their non-transgenic female littermates ($p<0.001$), which followed their maternal transgenic founder M.

By comparison, the mean body weight of transgenic males was similar to that of the non-transgenic male siblings from the age of 3 to 11 weeks, although the relative body weights of transgenic males did increase as the males grew older. At 12 weeks of age transgenic males had a mean body weight of 378.7 ± 10.4 grams, which was 11% greater than the mean body weight of 342.1 ± 14.5 grams of their non-transgenic male littermates (p=0.05, figure 4.6).

4.4.4.2 Growth of transgenic rats in D animal line

The growth rate of transgenic rats in D animal line is shown in figure 4.7. Both transgenic females and males grew at the same rate as their non-transgenic sex-matched littermates from 4 to 12 weeks of age in this animal line.

4.4.5 Expressing sites of the transgene

To determine whether differences in the amount of pGH protein produced and secreted into blood might be due to different tissue sites of expression of the foreign gene in different transgenic genotypes, pGH mRNA was measured in total RNA extracted from organs of 2 transgenic female rats in each animal line (D and M). Subsequently 50 µgs of total RNA was subjected to analysis by ribonuclease protection assay (RPA, see chapter 2.10).

Initially, a wide variety of tissues (liver, kidney, heart, spleen, intestine, muscle, uterus and ovary) from individual pGH expressing female rats in D and M animal line were analysed for pGH mRNA. These two animal lines each possessed different levels of blood-borne pGH protein (see chapter 3.4.4). A different distribution of the pGH mRNA was observed in the two animal lines, with the highest levels

observed in the liver and uterus of M animal line and intestine in D line (see figure 4.8). The signal present in other tissues was relatively low but it appeared that there were differences in the preferential sites of expression amongst the other tissues, with the pattern as follows:

M line:

uterus>liver>ovary>muscle=intestine>heart>spleen>kidney

D line:

intestine>liver>spleen>heart>uterus>muscle>kidney>ovary

The transgenic animals in D line exhibited striking differences in the transgene expression pattern (figure 4.8). The major site of expression was intestine (confirmed in four independent protection assays), followed by liver, spleen and heart.

Another four fast growing transgenic founder rats (G, H, J and K) have also been analysed for the production of the pGH mRNA (data not shown here). In these animals only liver, kidney, intestine and spleen were assayed. In all four animals the major site of expression was found to be the liver.

In summary, the major site of pGH expression in transgenic animals was the liver and uterus with high expression also observed in the intestine of D line. It appears that all animals also express low but significant levels of the pGH mRNA in various other tissues including kidney, heart, spleen, muscle and ovary.

4.4.6 Inducibility of the transgene expression

Because the human metallothionein promoter contains a metal-responsive element it was plausible that the hMTIIa/pGH transgenic

genotype characterised by low levels of foreign protein in plasma and normal spontaneous growth might respond to zinc-induction by increasing their secretion of pGH protein. Therefore, 9 offspring (6 non-transgenics and 3 transgenics) from a litter produced by mating female founder D with a non-transgenic male were provided with drinking water containing 25 mM $ZnSO_4$ for two weeks commencing when they were 14 weeks old. The levels of pGH protein in plasma before treatment, at the end of the treatment and two weeks after the treatment are shown in table 4.1. In non-transgenic rats plasma immunoreactive pGH protein varied from undetectable (<17 ng/ml) to 26 ng/ml. The transgenic rats had detectable plasma levels of immunoreactive pGH before exposure to zinc (40, 42 and 31 ng/ml). After two weeks of zinc treatment plasma pGH in these three rats rose by 42%, 65% and 100%, and remained elevated 2 weeks after zinc was withdrawn (table 4.1).

With the litter of F1 rats from founder M, apparently no induction of pGH expression was found in the only transgenic rat (M1.4) of that litter (table 4.2) and this observation was confirmed by further experiment with F2 rats in this family (data not shown here).

4.4.7 Effect of zinc concentration on inducibility of pGH

In order to test the effect of different zinc concentrations on circulating levels of pGH, 30 transgenic rats and 30 of their non-transgenic littermates, derived from F3 transgenic females by mating them with unrelated non-transgenic males, were used in this experiment (see chapter 4.2.5 for the details of experimental design). Table 4.3 summarises the circulating levels of pGH in the 5 groups of rats before and after the zinc water treatment. Apparently, all non-

transgenic rats in this experiment had pGH levels in plasma below the negative control. In the 5 mM zinc treated-group the mean level of pGH in plasma after the treatment remained the same as before. In 10, 15 and 20 mM zinc treated-groups the levels of pGH induction in plasma of transgenic rats were 100%, 100% and 40% respectively. The highest inductions were found both in 10 and 15 mM zinc treated-groups, both of which were 13 ng/ml (from 13 to 26 ng/ml in 10 mM zinc treated-group and from 12 to 25 ng/ml in 15 mM zinc treated-group). Interestingly, one non-transgenic rat in 15 mM group and 5 non-transgenic rats in 20 mM zinc treated group lost body weight rapidly when the treatment commenced. A close observation revealed that these rats were refusing to drink the zinc water. All the transgenic rats in this experiment lived healthily without any problem with the zinc water.

4.4.8 Effect of induced GH expression on growth

The absolute and relative weights of liver, kidney, heart, spleen and lung in transgenic vs non-transgenic female rats of D animal line at 12 and 30 weeks of age, after pGH expression was induced in transgenic rats by 10 mM zinc sulphate (see chapter 4.2.6 for the details of experimental design), are demonstrated in figure 4.10. At 12 weeks of age no differences in the absolute and relative weights of liver, kidney, heart, spleen and lung were observed between the three groups of rats, transgenics zinc-treated, non-transgenics zinc-treated and transgenics on normal drinking water.

However, at 30 weeks of age the mean absolute weights of liver, kidney, heart, spleen and lung in transgenic zinc-treated group were significantly increased compared to those in the non-transgenic zinc-

treated and transgenic with no zinc-treated group. But this was not reflected in the relative weights of these organs. The mean relative weights of liver, kidney, heart, spleen and lung in the three groups were all the same statistically although the ratio of liver/BW was relatively greater in transgenic zinc-treated group (figure 4.10, also see table 4.4 for the details of the body weights).

4.4.9 Effect of constitutive expression of pGH on growth

The mean absolute and relative weights of liver, kidney, heart, spleen and lung together with the body weights in transgenic female rats vs non-transgenic controls of M animal line at 12 and 30 weeks of age are demonstrated in figure 4.9. At 12 weeks of age the mean absolute weights of liver, kidney and heart in transgenic group were significantly increased compared to the values in the non-transgenic control group. However, the mean relative weights of liver, kidney and heart were the same between the two groups. The mean relative weights of spleen and lung in transgenic group were significantly decreased when compared to the value in non-transgenic control group (figure 4.9).

At 30 weeks of age the mean absolute weights of the organs examined together with body weight in transgenic group were all significantly increased compared to those in non-transgenic controls, but the mean relative weights of liver, heart and lung were the same between the two groups and the mean relative weight of spleen in transgenic group was significantly decreased compared to the value in non-transgenic control group (figure 4.9, also see table 4.4 for the details of the body weights).

4.5.DISCUSSION

Animals produced in this study contained between 1-130 copies of the transgene per cell as judged by DNA dot-blot analysis (data not shown). Of the expressing transgenic rats analysed, the same pattern of integration has been maintained in F1 of A, C, D and M founder animals. But low frequency of inheritance of the transgene integrations was obtained in breeding founder A and C. In the case of founder A (figure 4.1a), only 5 of 19 offspring contained the transgene in endogenous DNA and none of them expressed pGH in their blood. It is possible that founder A had more than one integration site, with functional copies being present at only one locus. The F1 generation appear not to have inherited the functional copies of the transgene at this locus from their expressing parent. This result is consistent with the observation of transgenesis using the same gene construct in mice (Michalska, Ph.D thesis, 1988). Further Southern analysis on DNA from this family would be necessary to determine the validity of this speculation. In the case of founder C, only a single female animal of 13 pups derived from breeding of this animal was found to be expressing pGH. Further breeding of the expressing F1 (C1.13) indicated that none of the 13 F2 rats expressed pGH in their blood. The reason for the lack of inheritance of the transgene is uncertain. It is possible that the transgene remained extrachromosomal instead of being permanently integrated to the endogenous DNA of founder rat C. Extrachromosomal occurrence and inheritance of transgene in mice (Rassoulzadegen, *et al.*, 1986; Sodo, *et al.*, 1990) and zebrafish (Bayer and Compos-ortega, 1992; Khoo, *et al.*, 1992) have been previously reported and similar results were also obtained in the previous studies of transgenic mice and pigs with the same gene construct (Michalska, 1988; French, 1991).

In the pre-experiment for production of transgenic rats in this study, 14 of 17 foetuses analysed were positive to PCR (data not shown), which could be assumed that some of the unreasonable high frequency of transgene integration is due to the extrachromosomal occurrence of the transgene in the endogenous DNA.

In the case of pedigree D and M, the same patterns of integration have been maintained in 5 and 7 generations respectively, 50% of offspring derived from transgenic rats bred with non-transgenic animals had the transgene integrated in the endogenous DNA and were expressing pGH in their blood, indicating the stability of the transgene in the germlines, following Mendelian ratio (figure 4.1 c and 4.2).

As illustrated in figure 4.5, the enhanced growth of expressing transgenic rats appears to manifest itself at weaning, consistent with the expected expression of the MTIIa promoter at these earlier time points in development (Andrew *et al.*, 1991). This result is particularly interesting in light of the recent finding that mRNA and protein for the rat GH receptor and BP are present in the early embryonic tissues of the rat (Garcia-Aragon *et al.*, 1992). In addition, a study by Palmiter *et al.* (1983), suggested that hGH was produced in MT-hGH transgenic mice from fetal stages, and that accelerated growth in these mice commenced from 16 to 22 days of age. In the present study body weights of young rats, transgenics vs non-transgenic littermates, were monitored daily and individually from postpartum day 15 to 34 with the first discernible enhancement of growth occuring in transgenic female rats expressing pGH between postpartum day 22 and 23.

There appears to be a threshold level of plasma pGH required to initiate noticeable enhancement of growth in the transgenic rats. The animals of the D line express no more than 25 ng/ml of pGH in their plasma at 4 weeks of age and all exhibit normal growth compared to non-transgenic controls, whereas transgenic animals in the M line expressing upwards of 40 ng/ml in plasma appear to achieve enhanced growth relative to their non-transgenic controls. It is feasible that this threshold is due to the sequestration of pGH by the rat GHBP present in excess in serum (Mannor *et al.*, 1991; Tiong *et al.*, 1991). In this transgenic model, GHBP would sequester the low levels of pGH present in transgenic animals of D family preventing enhanced growth via the GH receptor. Importantly, when the expression was enhanced by Zn^{++}, transgenic rats in the D line have shown a noticeable increase in size at 30 weeks of age compared to non-transgenic controls.

The enhanced growth of MTIIa/pGH transgenic rats observed in this study has also been observed in studies in which intact rats were administered recombinant rGH at several doses. Groesbeck *et al.*, (1987) administered rGH at either 0.4 mg/day, 1 mg/day or 5 mg/day to intact 4 week old female rats and observed body weight increases of up to 51% in rats treated with 1 mg/day and 73% increases with the 5 mg/day dose. These body weight increases ceased upon removal of the rGH. Blood borne levels of rGH rose to approximately 350 ng/ml with the dose of 1 mg/day and 1200 ng/ml after 20 days of treatment with the 5 mg/day dose. In the MTIIa/pGH transgenic rat of the M line, plasma pGH levels of 45 ng/ml corresponded with body weight increases in female transgenic rats of 50%, ranging from 41% to 62%. Further investigation of individual tissues indicated that the growth of liver, kidney and heart was also significantly increased in transgenic

females relative to their non-transgenic controls, which was in line with the results using rGH (Groesbeck *et al.*, 1987) and bGH (Byatt *et al.*, 1991).

As illustrated in figure 4.8, in transgenic animals of the M line examined by RNAse protection assay, the major site of pGH expression was the liver. It is not surprising therefore, that these faster growing animals possessed higher plasma levels of pGH. In addition, pGH mRNA was observed in a wide variety of other tissues such as intestine, uterus, ovary, kidney, heart, spleen and muscle, which is consistent with the housekeeping role of the MTIIa promoter (Durnam and Palmiter, 1981; Karin and Richards, 1982). The transgenic animals in the D line exhibited lower plasma pGH levels than the animals in the M line. Interestingly, transgenic animals exhibited higher expression of the transgene mRNA in the intestine than the liver in the D line (figure 4.8). Therefore, the lower levels of pGH expression in this animal line may be due to their lack of liver-specific expression of the transgene. This probably indicates that transgenic rats in this animal line are unique due to insertion of the transgene at a specific chromosomal location which is able to confer different regulation on the integrated sequences. Effects of the position of chromosomal integration site on expression of transgenes in tissues have previously been reported (Lacy *et al.*, 1983).

The plasma levels of pGH in transgenic animals from the D line have also been shown to be inducible by zinc (table 4.1) and the growth of these animals is also zinc inducible (figure 4.10). Similar anomalies between lines of expressing transgenic animals have been observed by Burdon *et al.*, (1991) who demonstrated differential hormonal regulation

of Whey Acidic Protein transgenes in three lines of transgenic mice. Since transgenic animals from the M line do not show expression changes in response to zinc (table 4.2), it seems likely that the induction phenomena may be a characteristic specific for the D line. Interestingly, with the treatment of zinc the animals in the D line showed a pattern of enhanced growth different from that of animals in the M line. Proportional enhancement of growth was observed in almost all internal organs examined in animals of the D line when the levels of pGH expression were induced by zinc, which is probably due to the intestinal expression.

In the zinc induction experiment, the levels of pGH expression in transgenic animals were induced up to 100% by Zn^{++} when the concentrations of zinc sulphate were over 10 mM in their drinking water. No increase in expression was observed in the animals treated with 5 mM Zn^{++}, which suggests that there may be a tissue threshold level of Zn^{++} which has to be exceeded before noticeable enhancement of pGH expression is initiated in these particular transgenic animals. Once zinc in the circulating system exceeds this level, maximum enhancement (100%) of the expression can be induced, and maintained in the animals during the rest of their life. This may be another intrinsic characteristic specific for the D line, although an examination of Zn^{++} concentration in rat blood is required to prove this hypothesis. Interestingly, in this study I observed that one non-transgenic rat treated with 15 mM Zn^{++} and 5 of the non-transgenic animals treated with 20 mM Zn^{++} refused to drink the zinc water, which was not observed in transgenic rats. The reason for this is uncertain at this stage.

In this study two phenotypically different transgenic animal lines have been selected from the 11 expressive transgenic rats produced by microinjection of the same DNA construct. One line appears to express pGH primarily in the liver, exhibit plasma pGH levels about 45 ng/ml and respond to the hormone with enhanced growth throughout their life. The second line is essentially indistinguishable from non-transgenic controls with respect to their growth and exhibit low levels of plasma pGH, but can be induced to express GH in the presence of Zn^{++} in their drinking water. Transgenic animals in this line showed a enhanced growth following the induction of expression of the transgene by Zn^{++}. Expression of pGH in these two animal lines appears to show a different tissue distribution. It is likely that these two group of animals reflect differences in the chromosomal location of the introduced transgene, although this suggestion will require substantiation by further experiment.

Table 4.1 Effect of zinc on levels of pGH expression of transgenic rats (D line)

Rat No.	Inherited Transgene	pGH before zinc (ng/ml)	pGH with zinc (ng/ml)	pGH after zinc (ng/ml)
D1.1	YES	40	66	60
D1.2	No	<17	25	<17
D1.3	No	19	<17	<17
D1.4	No	<17	20	<17
D1.5	No	<17	<17	<17
D1.6	YES	42	83	78
D1.7	YES	31	43	39
D1.8	No	20	<17	26
D1.9	No	20	<17	<17

Table 4.2 Effect of zinc on levels of pGH expression of transgenic rats (M line)

Rat No	Sex	Inherited transgene	pGH before zinc (ng/ml)	pGH during zinc (ng/ml)	pGH after zinc (ng/ml)
M1.1	male	No	<8	<8	12
M1.2	male	No	<8	<8	<8
M1.3	male	No	<8	<8	<8
M1.4	female	Yes	27	31	26
M1.5	female	No	<8	<8	<8
M1.6	female	No	<8	<8	<8
M1.7	female	No	<8	<8	10
M1.8	female	No	9	<8	<8

Table 4.3 Effect of zinc concentration on induction of pGH expression

Treatment	Genotype	n	pGH (ng/ml) before Zn^{++}	pGH (ng/ml) after Zn^{++}
Control	transgenics	6	15±1.4	14±1.1
	non-transgenics	6	<8	<8
5 mM	transgenics	6	14±1.2	15±0.8
	non-transgenics	6	<8	<8
10 mM	transgenics	6	13±1.3	26±2
	non-transgenics	6	<8	<8
15 mM	transgenics	6	12±1.1	25±1.8
	non-transgenics	5	<8	<8
20 mM	transgenics	6	15±1.2	21±1.6
	non-transgenics	1	<8	<8

Values are means ± s.e.m.

Table 4.4 Growth of transgenic rats (T) vs non-transgenic controls (NT) of both the D and M lines

				12 wks		30 wks	
Line	**Sex**	**Genotype**	**n**	**BW (g)**	**Relative size**	**BW (g)**	**Relative size[1]**
M	F	T	6	298±7.5***	1.41	514±10.7***	1.73
		NT	6	212±4.4	1.00	297±2.9	1.00
	M	T	6	387±10.2**	1.13	762±31.4***	1.39
		NT	6	344±4.3	1.00	550±19	1.00
D	F+zinc	T	6	217±6.6	0.95	417±5.6***	1.46
		NT	6	229±4.4	1.00	285±5.9	1.00
	F	T	6	223±6.7	1.02	312±10.7	1.06
		NT	6	218±5.8	1.00	294±7.6	1.00
	M+zinc	T	6	335±6.3	0.99	627±14.2**	1.14
		NT	6	340±6	1.00	550±11.3	1.00
	M	T	6	342±8.2	1.01	576±17.4	1.05
		NT	6	339±7.5	1.00	546±12.5	1.00

1. Mean body weight of transgenic animals in relation to the mean body weight of their non-trans

**** P<0.01.**

*****P<0.001.**

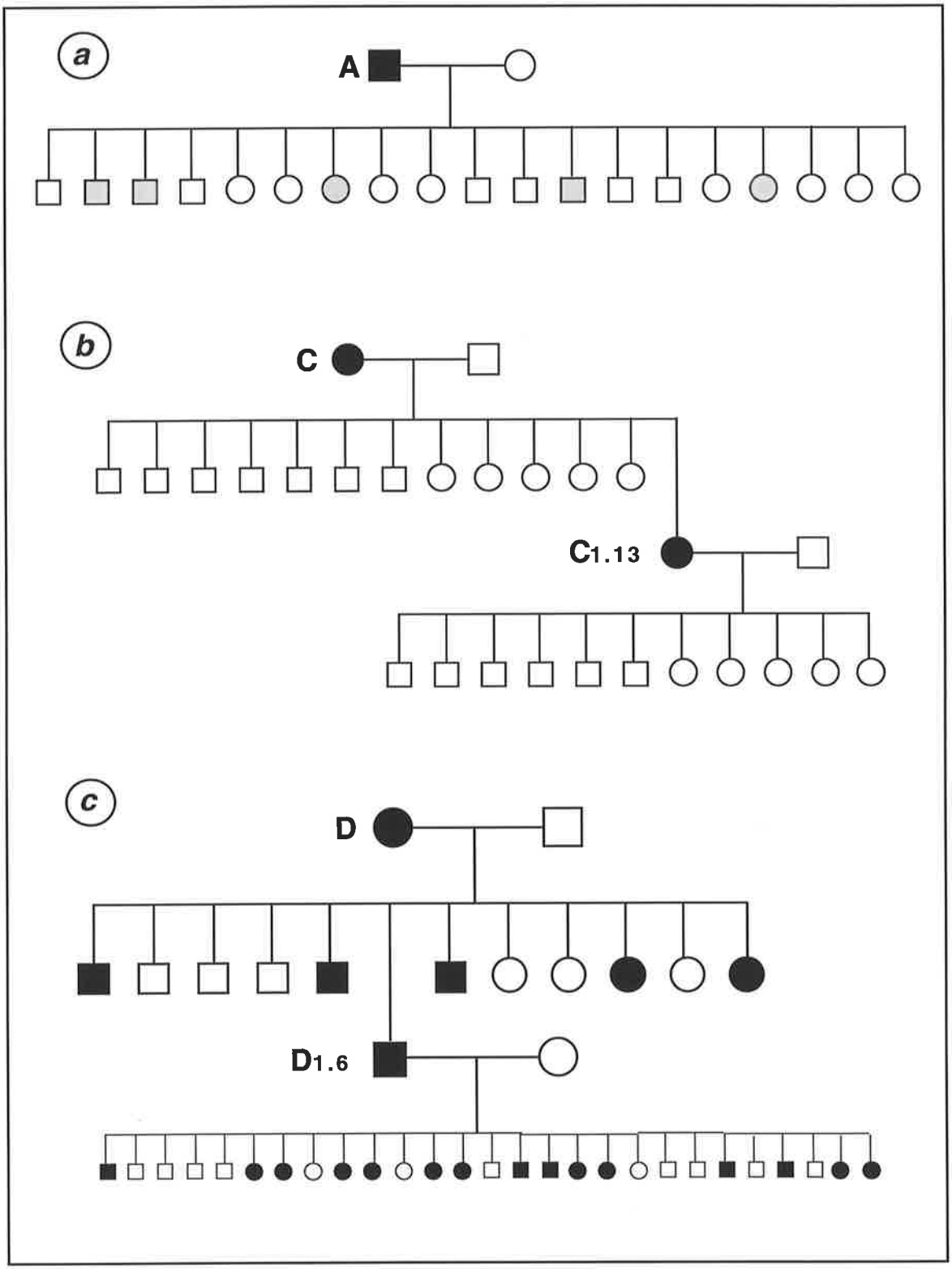


Figure 4.1 Pedigree analysis of MTIIa/pGH transgenic rats.

Rats expressing transgene are indicated by the solid symbols. Squares represent males and circles represent female rats. Shaded symbols represent the rats that carry the transgene but did not produce pGH in their blood.

Figure 4.2 Pedigree analysis of MTIIa/pGH transgenic rats for M animal line.

Rats expressing the transgene are indicated by the solid symbols. Males are shown as squares and females circles. All of the rats positive to PCR in this animal line exhibited high levels of pGH in their blood (also see figure 4.4 for the details of PCR results).

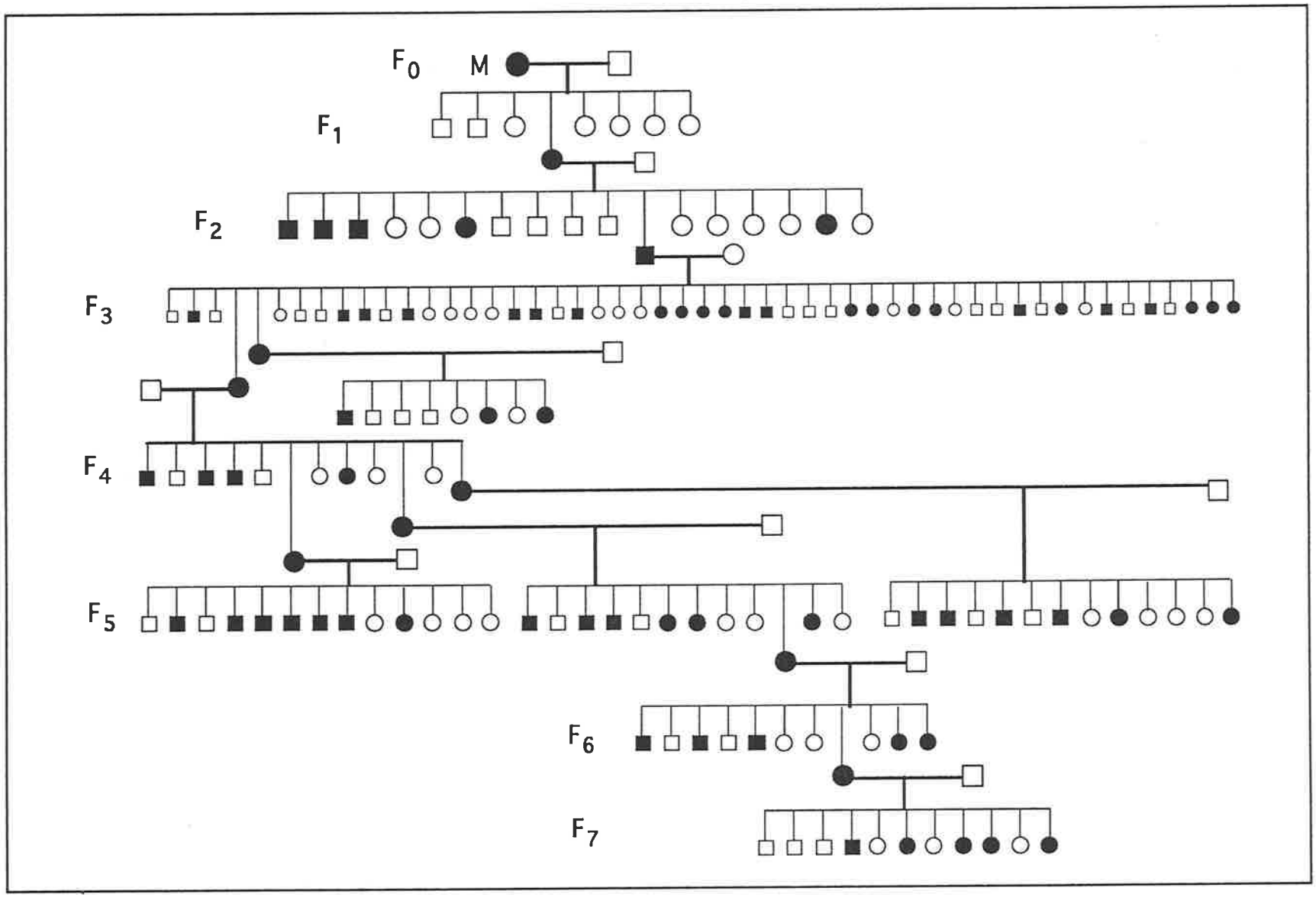

F0
M
F1
F2
F3
F4
F5
F6
F7

Figure 4.3 PCR identification of F_2 transgenic rats of the D line.

Polymerase chain reaction (PCR, see chapter 2.9 for details) was carried out on the F_2 generation of D line to quickly identify those offspring carrying the transgene. The two primers used were designed to amplify up a 130 bp portion of the human MTIIa promoter (Primers 1367 and 1384). PCR reactions were carried out in a total volume of 50 µls.

In this PCR one negative control, containing non-transgenic rat DNA (track **N**) and a positive control (track **P**) in this case DNA from Founder D were included. Tracks **1-27** contain DNA from rats **D1.6.1-D1.6.27**.

PCR Identification of F_2 transgenic rats of the D line

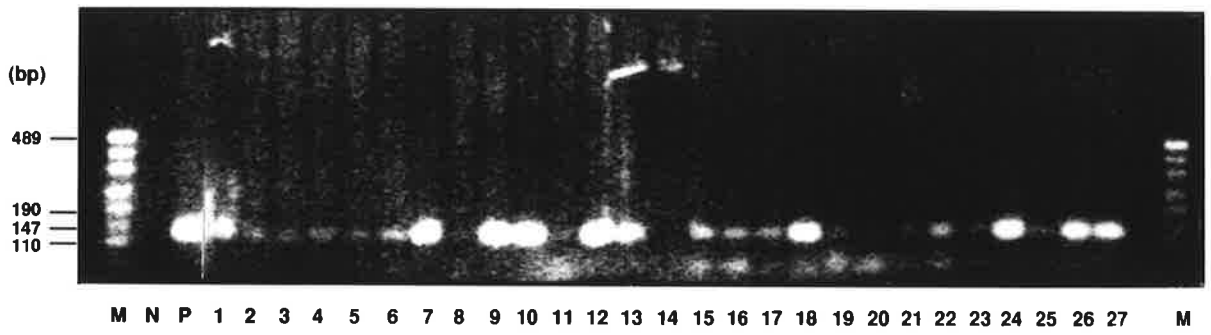
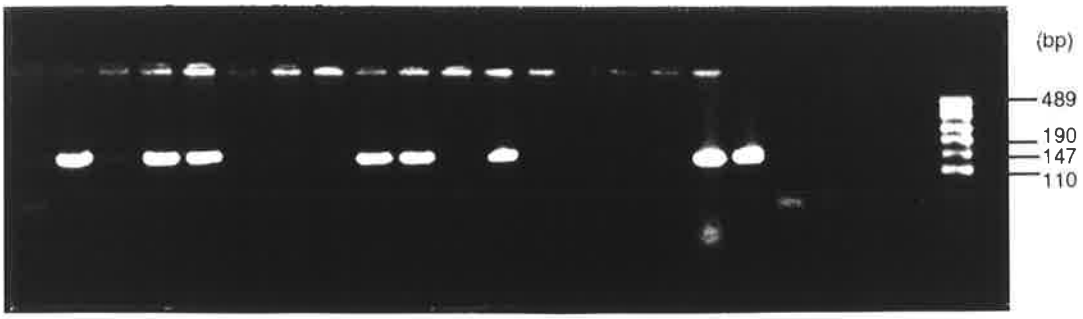


Figure 4.4 PCR identification of F_3 transgenic rats of the M line.

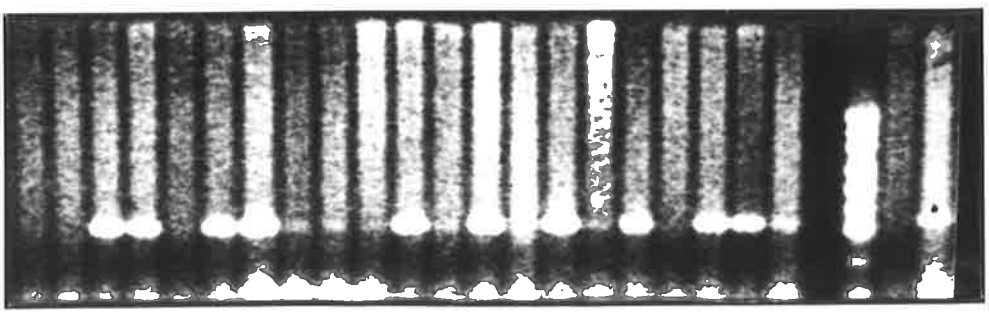
Three PCRs were carried out on the F_3 generation of M line to identify the transgenic rats. Tracks **N** represent negative controls and tracks **P** represent positive controls. **M** stand for DNA molecular weight Markers. Tracks **1-51** contain DNA from rats **M1.4.11.1-M1.4.11.51**.

PCR Identification of F_3 transgenic rats of the M line



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 P P N N M

N P M 17 18 19 20 21 22 23 24 25 26 27 28 29 30



31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 M N P

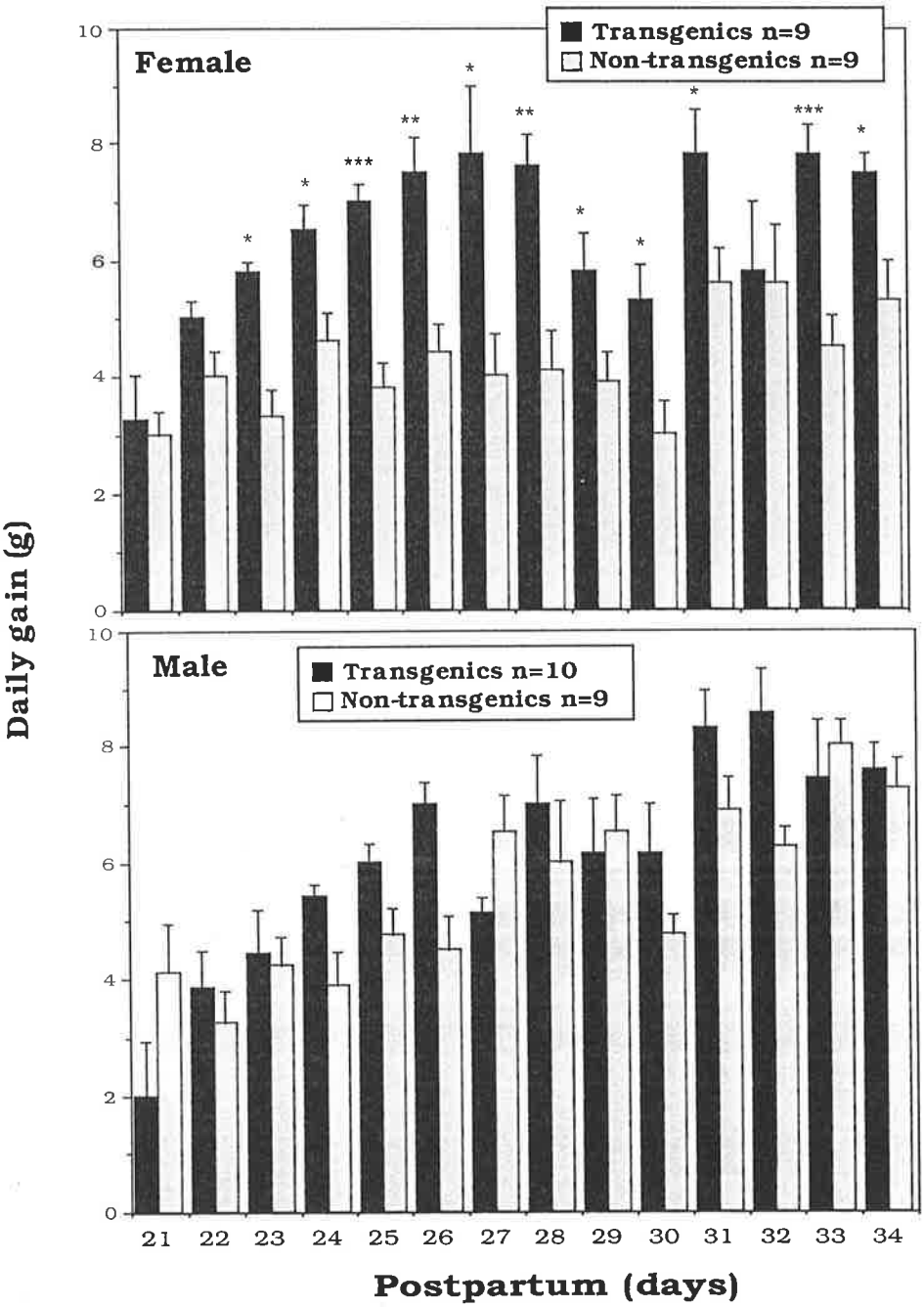


Figure 4.5 Daily gain of transgenic vs non-transgenic female or male rats of M line. Values are expressed as mean ± s.e.m.
*** p<0.05; ** p<0.01; *** p<0.001.**

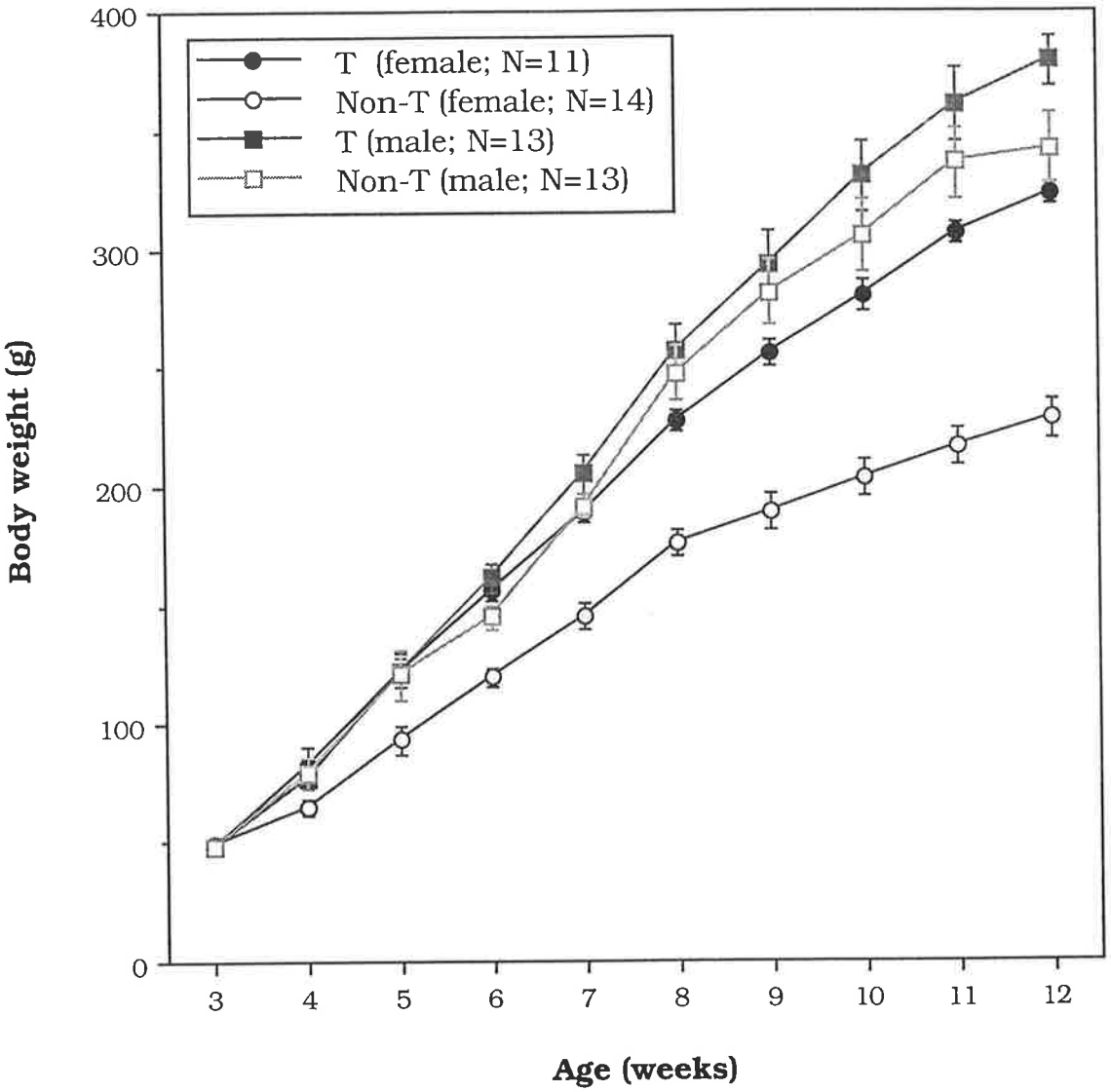


Figure 4.6 Growth rate of transgenic (T) vs non-transgenic (Non-T) female or male rats of M line. Values are expressed as mean ± s.e.m.

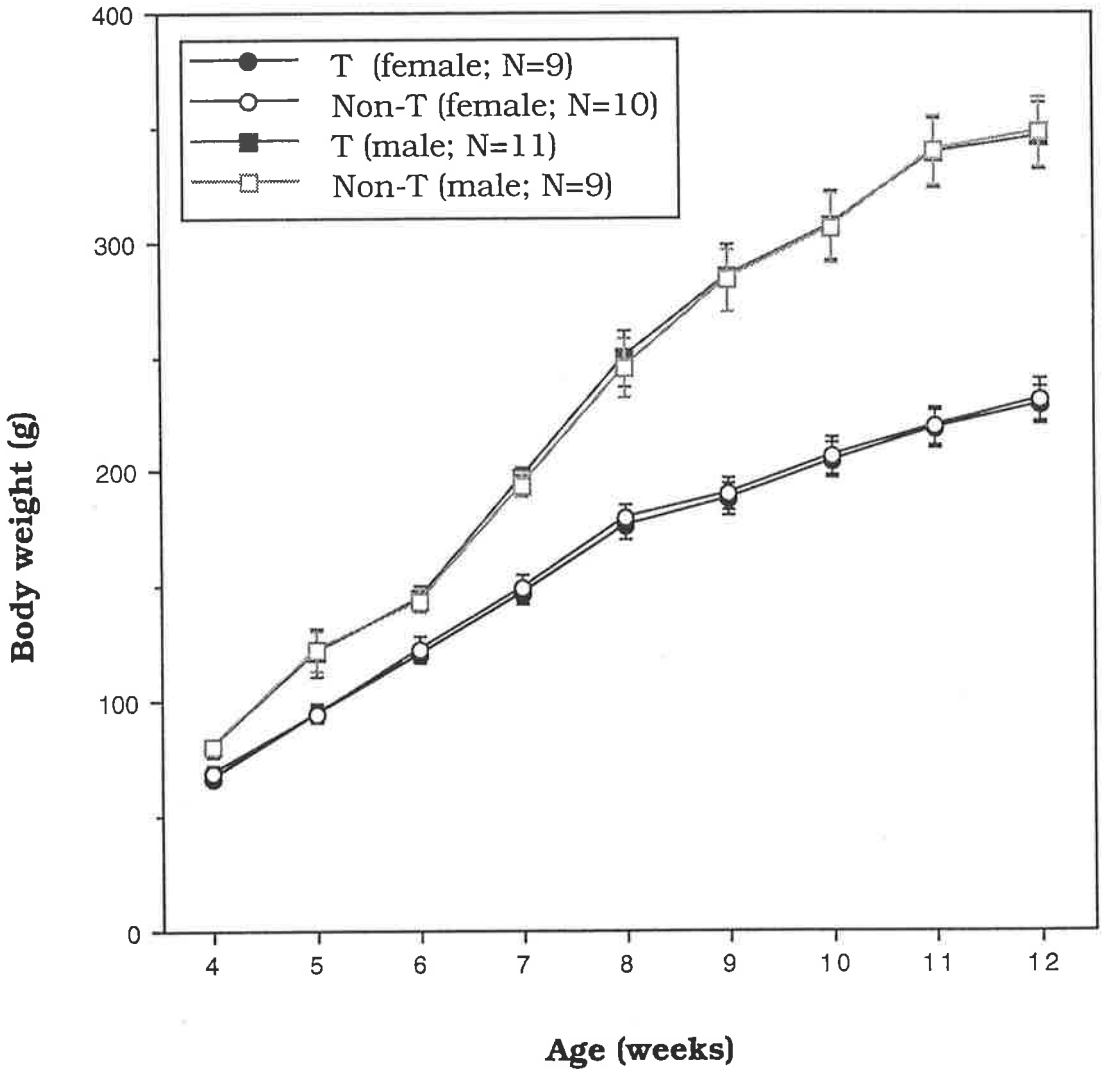
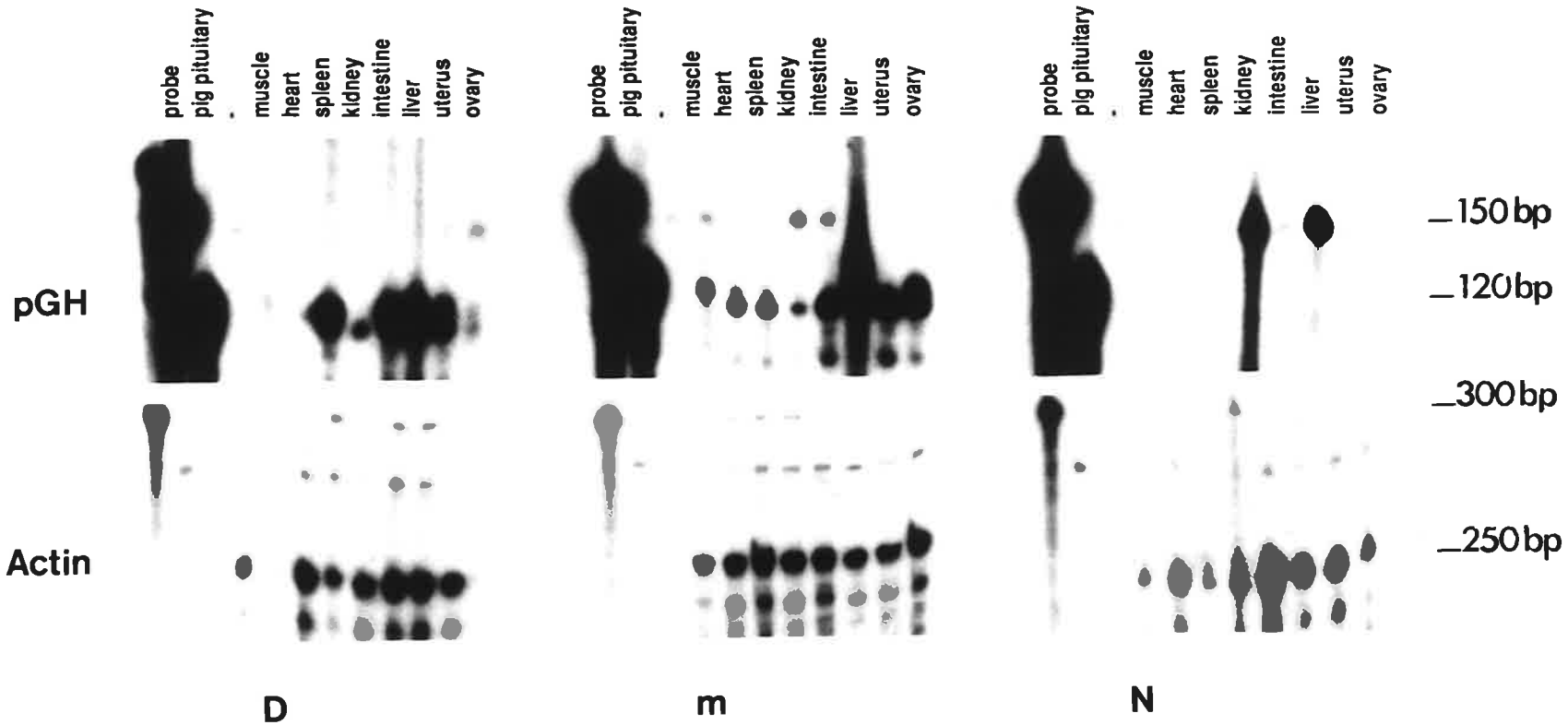


Figure 4.7 Growth rate of transgenic (T) vs non-transgenic (non-T) female or male rats of D line.
Values are expressed as mean ± s.e.m.

Figure 4.8 Expression of MTIIa/pGH transgene in different tissues of the two transgenic animal lines.

D represents the D animal line.
M represents the M animal line.


probe
pig pituitary
muscle
heart
spleen
kidney
intestine
liver
uterus
ovary
pGH
Actin
D
m
N
150 bp
120 bp
300 bp
250 bp

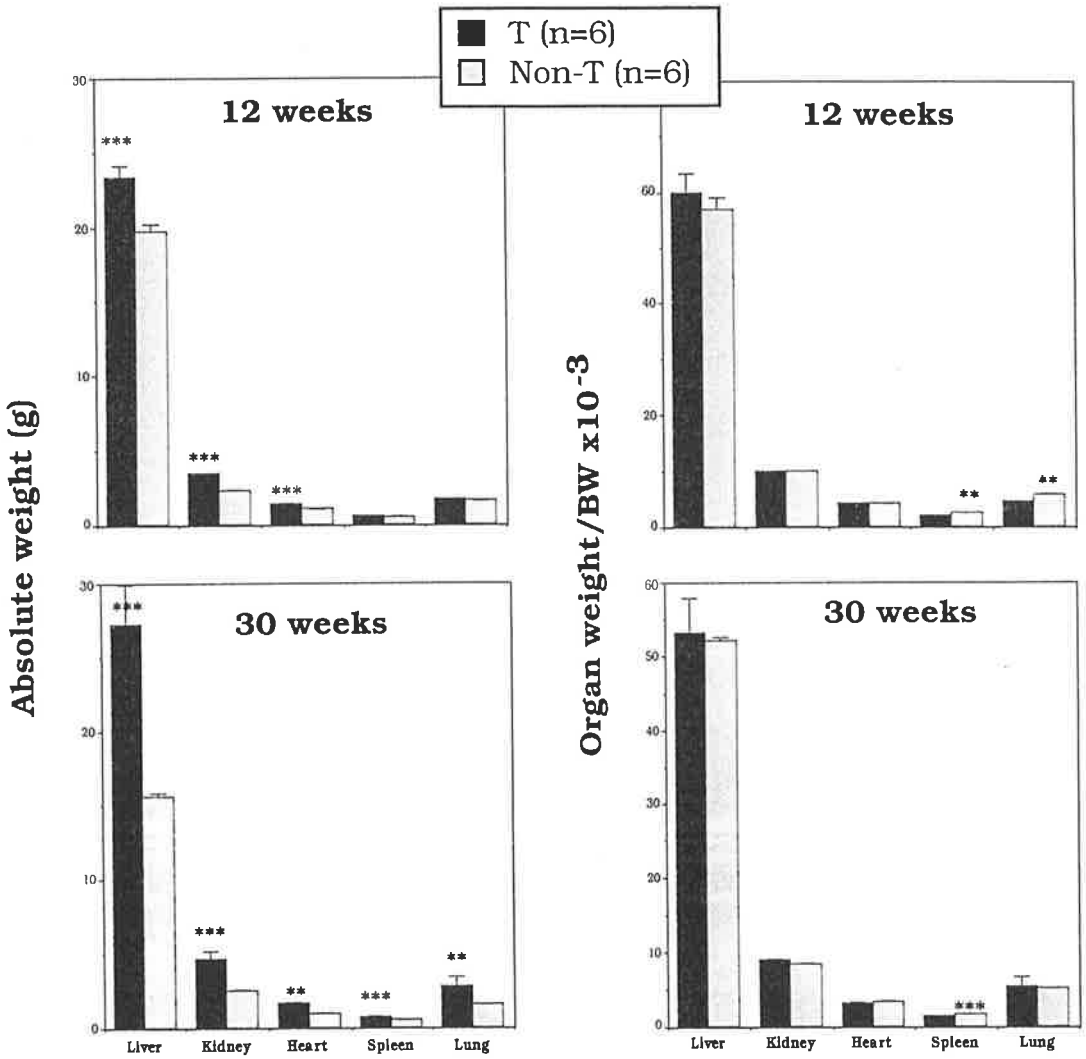


Figure 4.9 The absolute and relative organ weights of transgenic (T) and non-transgenic (non-T) female rats of M line. Values are expressed as mean ± s.e.m. **p<0.01; *** p<0.001

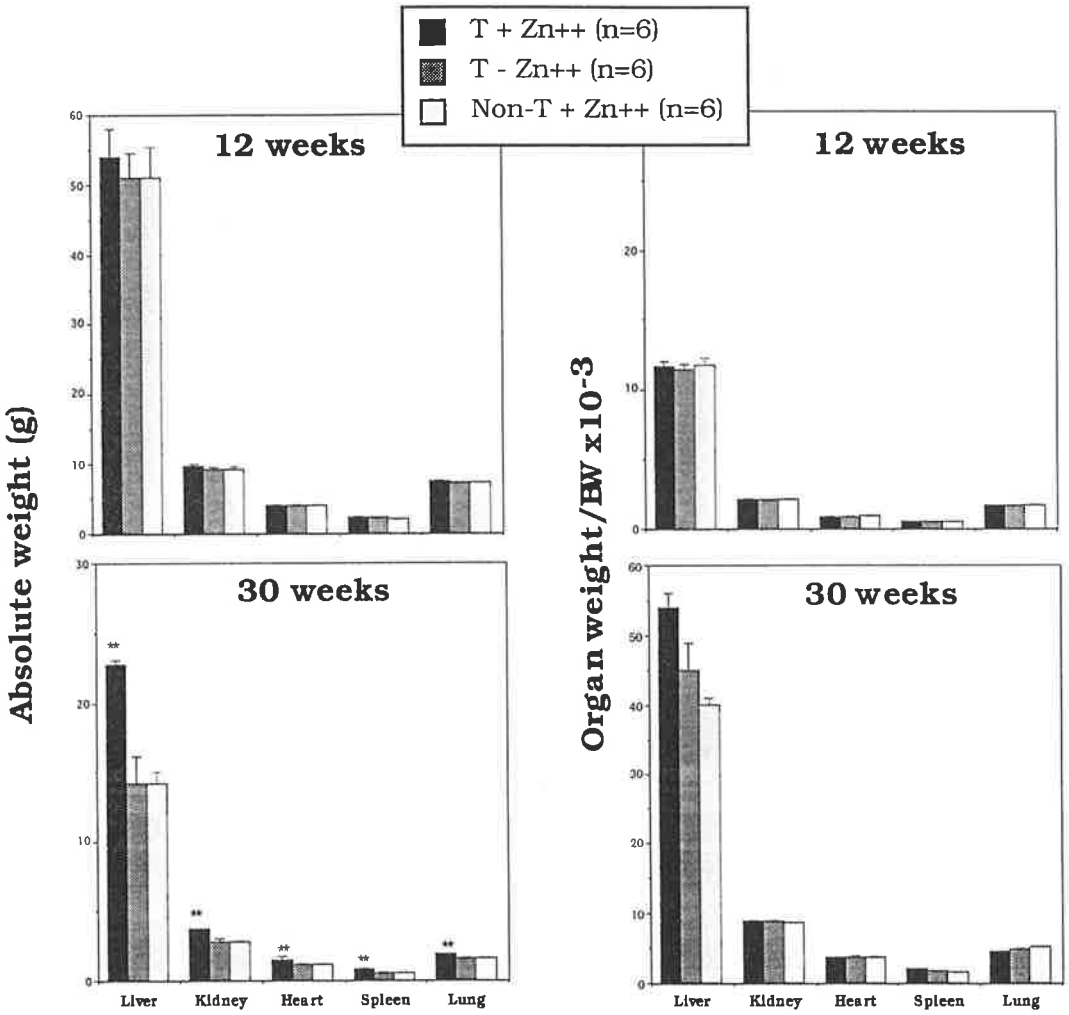


Figure 4.10 The absolute and relative organ weights of transgenic (T) and non-transgenic (non-T) female rats (D line) with or without Zn^{++} treatment.
Values are expressed as mean ± s.e.m.
**** p<0.01**

Chapter 5
Fertility of MTIIa/pGH female transgenic rats

5.1 INTRODUCTION

Some of the earliest studies to implicate GH as a modulator of gonadal function were carried out in teleosts (Higgs *et al.*, 1976; Young *et al.*, 1983; Fostier *et al.*, 1983). However, the claims of an association were dismissed for the most part, owing to the use of heterologous (mammalian) hormones in these early studies and the possibility of contamination with LH and FSH at the high dosages required to elicit a biological response. However more recently, Singh *et al.* (1988) have demonstrated that repeated administration of recombinant salmon GH can stimulate gonadal growth and elevate circulating sex steroid levels in hypophysectomized cat fish, Fundulus heteroclitus. Similarly, in the common carp GH has been shown to affect steroid production by vitellogenic and preovulatory goldfish ovarian follicles incubated *in vitro* and, importantly, potentiate the actions of common carp gonadotropin and human chorionic gonadotropin (hCG) on oestradiol-17β (E**2**) production by the ovarian tissues (Van Der Kraak *et al.*, 1990).

One of the first studies in mammals to implicate GH in ovarian function was carried out in rats by Advis *et al.* (1981). These researchers observed an enhanced output of progesterone (P) by ovarian tissue *in vitro* in response to both hCG and human FSH if the ovaries were subject to pretreatment with GH *in vivo.* Subsequently, Lanzone *et al.* (1990) found that treatment with homologous GH (1 mg/ml) stimulates basal P biosynthesis by highly luteinized human granulosa cells in culture. Importantly, the combination of generally noneffective doses of hCG (6 and 12 ng/ml) and human GH (250 and 500 ng/ml) have been shown to enhance P production to levels comparable with those seen with the highest doses of human GH or hCG alone,

indicating a synergistic effect. Moreover, JØrgensen *et al.* (1989) observed a synergistic action between human GH and FSH on the ovaries of hypophysectomized rats , resulting in a greater number and a larger size of follicles. JØrgensen *et al.* (1991) also found that daily treatment of rats with 1.0 and 3.3 IU/kg of human GH for a 2 week period prior to matings produced a significant prolongation of the oestrous cycle, causing the number of mating days to be more than doubled compared with placebo-treated controls. The GH treatment also significantly increased the number of implantation sites and corpora lutea in human GH-treated rats relevant to placebo-treated controls.

In studies with mice model, transgenic with GH fusion genes, the data is conflicting. In transgenic mice expressing the human and porcine GH gene, the male mice were fertile but the females were found to be sterile. In contrast, female mice expressing the bovine and ovine GH retained their fertility (Mayerhoffer *et al.*, 1990; Orian *et al.*, 1989). Subsequent studies (Mayerhoffer *et al.*, 1990) revealed that in sterile females the ovaries, although not necessarily enlarged, had been adversely affected by chronic exposure to the heterologous GH. Human GH, which in the mouse has additional lactotrophic activity, produced complex alterations including the acceleration of follicular development, increased atresia, and massive degeneration of interstitial cells. By contrast with bovine GH, which in the mouse exhibits isolated somatotrophic activity, the morphological signs of atresia reduced. In the transgenic GH mice, it has been speculated that there is an accelerated aging of the mouse ovary possibly due to the combination of somatotrophic and lactotrophic activity of human GH (see review, Katz *et al.*, 1993). In view of our growing interest in the physiological

consequences of porcine GH transgene expression, the present study was undertaken to investigate the reproductive sequelae of enhanced porcine GH expression with the transgenic rat model.

5.2 MATERIALS AND METHODS

5 2.1 Animals

Transgenic founder D and M were mated by fertile non-transgenic males. Two transgenic animal lines were established from these two founders. Offspring from the third to the fourth generations of D animal line and from the third to the seventh generations of M animal line were used for these studies. The D animal line was located in CSIRO Animal House Glenthorne, SA, whilst the M animal line was housed in Animal Resource Centre at the University of Adelaide. The conditions of maintenance have been described previously (see chapter 2.1).

5.2.2 Assessment of puberty onset

Immature female rats, including 22 transgenic and 24 non-transgenic littermates, obtained from 8 litters, born to transgenic mothers of the M line following mating to unrelated fertile non-transgenic males, were used in this study. Beginning at 28 days of age the pups were examined daily between 1000-1200 hour for signs of vaginal opening and the timing of onset of puberty assessed by the occurrence of vaginal patency.

5.2.3 Assessment of oestrous cycle

Vaginal smears were taken daily from 12 mature transgenic female rats and 12 non-transgenic littermates of the M line for 5 cycles

starting at 10 weeks of age. These animals were housed in all-female groups, 4 animals per cage, from the time of weaning and throughout the period of taking vaginal smears. The presence and abundance, as determined subjectively, of live (nucleated) epithelial cells, cornified epithelial cells, and leukocytes were recorded. Ovulation was verified by a conversion of a purely conified (oestrous) smear to a mestestrous smear (mixed epithelial cells and leukocytes). The day of ovulation itself was defined as the first day of a cornified smear.

5.2.4 Identification of transgene

The transgenic status of all rats used in these studies was confirmed by PCR carried out on DNA samples isolated from tail tissue taken at 4 weeks of age (see chapter 2.9 for details).

5.2.5 Identification of transgene expression

Blood samples were taken from tail vein at 4 weeks of age for porcine GH radioimmunoassay. Rats with GH levels higher than negative control samples (>8 ng/ml) were considered to be expressing transgenics (see chapter 2.11 for details).

5.3 EXPERIMENT DESIGNS

5.3.1 Comparison of ovulation

To examine the effect of GH expression on ovulation rate, tail tissue and blood samples were taken at 28 days of age from the fourth generation of animals of the D line. Nine transgenic immature female rats, which were both positive to PCR and porcine GH RIA, and 9 of their non-transgenic littermates, which were both negative to PCR and porcine GH RIA, were identified and selected for this investigation. 10

IU PMSG was injected to each of these rats at 32 days of age, followed 48 hours later by 10 IU hCG injection. 24 hours after hCG injection the rats were killed by cervical dislocation and the number of oocytes released was determined (see chapter 2.4 for details).

5.3.2 Comparison of implantations

To compare implantation rates, 16 transgenic female rats and 16 non-transgenic littermates from the third to the fourth generation of the D line were mated at 12 weeks of age with 4 unrelated fertile non-transgenic males purchased from the main animal colony of CSIRO, Glenthorne. To enhance GH expression, 8 of the transgenic and 8 of the non-transgenic littermates were exposed to 10 mM zinc sulphate for 3 weeks prior to mating, whilst the remaining 8 transgenic and 8 non-transgenic littermates remained on normal drinking water. Matings were established by placing one transgenic and one of her non-transgenic littermates with a single male. During mating, vaginal plugs were checked daily for first week. 14 days after a vaginal plug was found, females were removed from the males when pregnancy was indicated by increased body weight. Non-mated females were not used in this study.

In a second experiment, 8 transgenic female rats and 8 non-transgenic littermates from the third to the seventh generation of M line animals were selected. All the female rats were mated with 3 unrelated fertile non-transgenic males of the same strain purchased from Animal Resource Centre, Adelaide University. Matings were established and pregnancy was determined in the way described above.

At birth the following parameters were recorded: litter size, number of pups, number of surviving pups and body weight. At weaning the numbers of surviving pups were counted, and mothers sacrificed to determine the total number of implantation sites.

5.3.3 Assessment of weights of uterus, ovary and pituitary

To investigate the effect of porcine GH expression on growth of reproductive organs and glands, comparison was made between 12 transgenic rats of the D line exposed to 10 mM zinc water for a 3 week period starting at 9 weeks of age, and 12 transgenic and 12 non-transgenic littermates which remained on normal drinking water. At 12 weeks of age, 6 of the rats exposed to zinc water and 6 of the transgenic rats held on normal drinking water plus 6 non-transgenic littermates were sacrificed for assessment of organ weights and the remaining 18 rats sacrificed at 30 weeks of age.

A second, similar experiment was carried out using 12 transgenic and 12 non-transgenic littermates from the M line. 6 transgenic rats and 6 non-transgenic littermates were sacrificed at 12 weeks of age and remaining 12 rats at 30 weeks of age.

The body weight and the weights of uterus, ovary and pituitary, and liver, kidney, heart spleen and lung (see chapter 4.4.8 and .9) were recorded.

5.3.4 Assessment of lactation

To assess the effect of porcine GH expression on lactation 8 transgenic female virgin rats and 8 non-transgenic littermates of M animal line were mated with unrelated non-transgenic fertile males and

the pregnancy allowed to progress to term. The birth weights of all pups born were recorded and litter sizes reduced to 12 pups on day 3 after parturition.

To assess milk production each mother was separated from her pups at 10 o'clock in the morning on postpartum day 7 and day 10 to permit milk to accumulate in the mammary glands, and to permit digestion of milk previously obtained by the pups. 5 hours later the pups were replaced and permitted to suck for 30 minutes. No litter was removed from the cage nor was the milk yield determined until each litter had been nursed a total of 30 minutes. Body weights of the pups were recorded to the 0.1 g before and after the 30 minutes sucking. At 21 days of age when the babies were weaned, weights of litters were examined.

Litters in which any babies died before weaning were omitted from the study.

5.4 STATISTICAL ANALYSIS

All the data are presented as means $\pm$ s.e.m. Statistical differences between the two groups (transgenic and non-transgenic control) in the age of puberty, length of oestrous cycle, number of days which matings occurred, number of oocytes ovulated, litter size, the number of implantations and viable pups at weaning, milk production and the weights of uterus, ovary and pituitary were tested by Student's *t*-test. Comparison of zinc effect on implantation and litter size in treated and control of both transgenic and non-transgenic groups were

analyzed by ANOVA (SAS Institute Inc.,1988). Comparisons with $p>0.05$ were not considered significant.

5.5 RESULTS

5.5.1 Fertility studies in female rats of the M line

5.5.1.1 Onset of Puberty

The onset of puberty, evidenced by occurrence of vaginal patency, was delayed in female transgenic rats of the M line , compared to their non-transgenic littermates (table 5.1). Mean age at occurrence of vaginal patency was 36.2 $\pm$ 0.66 days (n=22) for transgenic rats expressing porcine GH, with a range from 31 to 43 days, and 34.4 $\pm$ 0.5 days (n=24) for non-transgenic littermates, with a range from 30 to 40 days. These means were significantly different from each other ($p<0.05$). The mean body weight on puberty in transgenic group was 122.5 $\pm$ 2.1 grams, which was significantly increased over the mean of 97.3 $\pm$ 2.7 grams found in the non-transgenic control group ($p<0.001$, table 5.1).

5.5.1.2 Oestrous cycle

Examination of vaginal smears indicated that 10 of 12 transgenic females had normal oestrous cycles with an average length of 4.6 $\pm$ 0.2 days. Of the 2 remaining transgenic rats, 1 had an apparent pseudopregnancy (12 day interval between consecutive transitions from leukocyte-containing to leukocyte-free smears) and 1 had no evidence of either cycle or pseudopregnancy. Of 12 control non-transgenic female rats, 10 had normal oestrous cycles with an average length 4.5 $\pm$ 0.3 days with the 2 remaining rats showing a 11 days interval indicitative of pseudopregnancy. There was no significant difference between the oestrous cycles of the two group of rats.

5.5.1.3. Matings

The numbers of rats mated per day are shown in table 5.2. Of the 8 transgenic female rats used in this study 4 were mated during the first cycle (two on day 3 and two on day 4), 4 mated within the second cycle (two on day 9 and two on day 10). The mean number of mating days for female transgenic rats of the M line was 6.5 $\pm$ 1.2. While the mean number of mating days for non-transgenic controls was 5.1 $\pm$ 0.8, ranging from 2 to 9 days. Number of the mating days for transgenic female rats of the M line was similar to that of their non-transgenic littermates (figure 5.1).

5.5.1.4. Pregnancy

The pregnancy data for transgenic rats of M line are shown in table 5.3. The results of the studies revealed that the female transgenic rats expressing the MTIIa/pGH construct were fertile, and that the mean number of implantation sites in transgenic group was significantly higher than that of their non-transgenic littermates (15.3 $\pm$ 0.35 and 12.7 $\pm$ 0.82 respectively, $p<0.01$). However, the expression of this transgene had no effect on the mean litter size (13.5 $\pm$ 0.53, including 7 dead pups, in transgenic group compared with 12.4 $\pm$ 0.77 in non-transgenic control group, $p>0.05$) and the mean numbers of viable pups at weaning were comparable between the two groups (11.9 $\pm$ 0.56 in transgenic and 11.4 $\pm$ 0.45 in non-transgenic controls, $p>0.05$). Embryonic loss, as measured by comparing the mean number of viable pups at birth and weaning to the mean number of implantations in transgenic group, was significantly enhanced ($p<0.05$).

5.5.1.5 Lactation

The milk ejection data are shown in table 5.4. The mothers nursed uninterruptedly and usually had completed nursing their pups by the end of the 30-minute nursing period. The transgenic lactating rats yielded an average of 2.68 g of milk on postpartum day 7 and 4.68 g on day 10, compared to an average of 2.03 g on day 7 and 2.28 g on day 10 which their non-transgenic littermates produced to their 12 pup litters when suckled for 30 minutes under relatively undisturbed conditions. The milk ejected by transgenic lactating rats on postpartum day 10 was significantly greater than that by non-transgenic controls ($p<0.05$).

The mean birth weights of 5.51 $\pm$ 0.04 g in transgenic and 5.55 $\pm$ 0.06 g in non-transgenic controls were similar, as were the live litter weights at day 7 and day 10 in the two groups (159.7 $\pm$ 8.3 and 224.3 $\pm$ 6 in transgenic group, compared to 168.3 $\pm$13.5 and 222.9 $\pm$ 7.5 in non-transgenic controls at day 7 and day 10 respectively). However, the mean litter weight of 499.9 $\pm$ 10.9 g in transgenic group at day 21, was significantly greater than the mean of 444.1 $\pm$ 16.1 g for the non-transgenic littermates (see table 5.5, figure 5.2).

5.5.1.6 Growth of uterus, ovary and pituitary

The absolute and relative weights of uterus, ovary and pituitary in transgenic female rats vs non-transgenic controls of the M line at 12 and 30 weeks of age are shown in figure 5.3. At 12 weeks of age the mean absolute weight of uterus and ovary in transgenic group were significantly greater than the values in non-transgenic control rats, but this was not reflected in the pituitary weight. Interestingly, the mean absolute weight of pituitary in transgenic group was significantly lower

than non-transgenic littermates ($p<0.001$). The weight of uterus in relation to body-weight of transgenic animals remained the same as that in non-transgenic controls. However, the relative weights of ovary and pituitary in transgenic females were very significantly lower than the values in non-transgenic controls ($p<0.001$, see figure 5.3).

At 30 weeks of age the absolute weights of uterus and ovary in transgenic group were significantly increased compared to those in non-transgenic controls, but this was not reflected in pituitary. However, the weights of uterus and ovary relative to the body weight in the two groups remained the same whilst the relative weight of pituitary in transgenic females was significantly lower than that for non-transgenic controls ($p<0.001$, see figure 5.3).

5.5.2 Fertility studies in D animal line

5.5.2.1 Ovulation rate in immature transgenic rats

All the rats responded well to the PMSG and hCG treatment with the results shown in table 5.6. The mean number of oocytes recovered from the transgenic group was 34.6 ± 1.9, which was significantly higher than the mean number, 24.7 ± 1.3 recovered from the non-transgenic littermates ($p<0.001$). The difference was so pronounced that only two rats in transgenic group had an ovulation rate lower than 30 (25 and 28), which were overlapped with the number of oocytes recovered in the control group. In the transgenic group no interrelationship was found between the levels of porcine GH and number of oocytes recovered.

5.5.2.2 Matings

The numbers of rats mated per day are shown in table 5.7. 30 of 32 rats, including 16 transgenic and 14 non-transgenic littermates, were mated within 7 days. Another two rats, one in non-transgenic Zn^{++} - group and one in non-transgenic Zn^{++} + group, failed to mate within the two week period allocated. They were replaced by 2 other rats from the same litters and mated within 7 days. For mating period no statistical difference was found between the groups of transgenic and non-transgenic controls (figure 5.4).

5.5.2.3 Pregnancy

The group mean pups born, body weights of the pups at birth, number of implantations and number of the viable pups at weaning are shown in table 5.8. The pregnancy rate was 100% in all the four groups. As in the M line, although the mean number of implantation sites in transgenic Zn^{++} - group was significantly increased compared to that in non-transgenic Zn^{++} - group (16.8 ± 1.06 vs 13.4 ± 0.81, $p<0.05$), no differences were evident in mean number of pups born (14.6 ± 1.2 and 12.6 ± 0.8 transgenic vs non-transgenic Zn^{++} - group respectively). However, the highest mean litter size (15.3 ± 0.9) was found in transgenic Zn^{++} + group, which was significantly increased compared to that in non-transgenic Zn^{++} + group (12.9 ± 1.0, $p<0.05$), although the mean number of implantation sites was not significantly different between these two groups (15.5 ± 0.9 and 13.4 ± 0.81 respectively). The number of viable pups at weaning was 13.8 ± 0.5 in transgenic Zn^{++} + group, which was significantly greater than that in all other three groups ($p<0.05$).

No significant differences were found in mean birth weights among the four groups, viz 5.6 ± 0.05 g in transgenic Zn++ + and 5.7 ± 0.09 g in non-transgenic Zn++ + group, and the mean birth weights were 5.4 ± 0.09 g in transgenic Zn++ - and 5.6 ± 0.13 g in non-transgenic Zn++ - group respectively.

When the two groups of transgenic were considered as one, implantations, litter size and viable pups at weaning in transgenic rats were all significantly increased compared to values in the non-transgenic controls (table 5.9).

5.5.2.4 Growth of uterus, ovary and pituitary

The absolute and relative weights of uterus, ovary and pituitary in transgenic vs non-transgenic control female rats of D animal line at 12 and 30 weeks of age are summarised in figure 5.5. At 12 weeks of age no differences in the absolute and relative weights of uterus, ovary and pituitary were observed between the three groups of rats, transgenics zinc-treated , non-transgenic zinc-treated and transgenics on normal drinking water.

However, at 30 weeks of age the absolute weights of uterus, ovary and pituitary in zinc treated transgenic group were significantly increased compared to those in non-transgenic zinc-treated and the transgenic no-zinc-treated groups, but this was not reflected in the relative weights of these organs and gland. The relative weights of uterus, ovary and pituitary in the three groups were all the same statistically.

5.6 DISCUSSION

A permissive action of GH has been clearly suggested by observations in GH-deficient mice (Snell, 1929) and human (Tanner & Whitehouse, 1975) where systemic provision of GH replacement therapy promoted follicular development (Bartke, 1964). Moreover, partial suppression of endogenous GH secretion in female rats resulted in delayed puberty and GH replacement in the rats under study was shown to increase ovarian P production in response to gonadotropins and to restore the normal timing of pubertal onset (Ramaley & Phares, 1980). However, Groesbeck *et al* (1987) observed that the onset of puberty was delayed an average of 2.7 days in the female rats treated with 1 mg rGH/day. A similar delay of 1.8 days has been found in the present studies in the female rats expressing the MTIIa/pGH transgene. These data are consistent with the observations previously made by Groesbeck *et al.*(1987) but the cause of the delay in the onset of puberty is uncertain. However, it is possible that the dramatic physiological changes created by the chronic overproduction of GH constituted a strong, nonspecific stress, which interferes with normal physiological and endocrine regulatory mechanisms. In any case, these results confirmed the finding obtained by Groesbeck *et al.* (1987) that excessive production of GH may be a cause of delayed onset of puberty in female rats.

JØrgensen *et al.* (1991) observed that treatment with human GH at the dose of 3.3 IU/kg per day for 29 to 76 days in female rats caused a significant prolongation of the oestrous cycle. Naar *et al.* (1991) demonstrated that expression of human GH•V and bovine GH genes in transgenic mice was associated with various degrees of impairment of

female reproductive functions, including lack of oestrus as seen in hGH •V transgenic mice and lengthening of the mating period as seen in both hGH•V and bGH transgenic female mice. In the present study with the M line, animals which expressed porcine GH, resulting in circulatory plasma levels of about 40 ng/ml, showed enhanced growth and had oestrous cycles similar to the non-transgenic littermate controls (4.6 ± 0.2 days vs 4.5 ± 0.3 days). Furthermore, the expressing transgenic females in the D line, with plasma porcine GH levels of 18 ng/ml and 40 ng/ml following exposure to zinc, had a similar mating period to their non-transgenic littermates. Taken together, this study clearly demonstrates that, unlike transgenic mice, transgenic female rats expressing porcine GH are fertile.

Although prolactin is the major galactopoietic hormone in the rat, GH clearly plays an important role in milk production (Madon *et al.*, 1986). When milk synthesis is arrested completely by suppression of endogenous prolactin and GH action, exogenous GH has been shown to restore milk yield to 50% of normal (Flint *et al.*, 1992). In the present study, the data gained with M line transgenic rats clearly demonstrated that chronic over-production of exogenous GH enhanced milk yield and increased the growth of new born rats during lactation. The extra GH failed, however, to sustain the high output of milk seen early in the lactation.

The present studies also show that expression of foreign GH transgene can enhance the number of ova ovulated following standard superovulation regimes. The mean number of ovulations in transgenic rats was 40% increased compared to non-transgenic littermates. Interestingly, all the immature transgenic female rats in the experiment

responded well to the treatments. The data are consistent with the findings of an *in vivo* interaction of human GH and FSH resulting in a synergistic effect on the number and size of follicles in the ovaries of young hypophysectomized rats (JØrgensen & Nowak 1989). Pretreatment of heifers with bovine GH has been shown to enhance superovulatory responsiveness to PMSG, leading to a significantly increased number of ovulations and of eggs recovered, and a reduction in the number of animals showing a poor ovulatory response. Furthermore, as shown by Gong *et al.* (1993) the increase is apparently due to the increase in the number of small follicles induced by GH pretreatment to undergo continued growth up to the day of ovulation. In the present studies, expression of porcine GH in transgenic rats from both D and M lines showed a significantly higher rate of implantations, strongly suggesting that expression of porcine GH in transgenic rats at the circulating levels of 20 and 45 ng/ml can enhance follicle growth or development, resulting in enhanced ovulation rates. Interestingly, higher number of viable pups was found both at birth and at weaning in transgenic rats of the D line, when the rats were exposed to zinc to increase the expression of the fusion gene resulting in plasma levels of 41 ng/ml. However, this increase was not showed in transgenic rats of the M line which expressed the transgene constitutively at the level of 45 ng/ml. This possibly means that expression of exogenous GH in rats at level of 20 ng/ml in plasma is sufficient to induce follicle growth up to ovulation.

When the circulating levels of porcine GH were increased by drinking zinc water at 9 weeks of age, the reproductive systems were mature and the rats tolerated better the high level of exogenous GH, with no adverse effects on the implantation rate. The increased number

of viable pups at weaning provides support of the view that chronic overproduction of porcine GH can enhance milk yield produced by lactating rats. This is also supported by the data obtained in the milk production experiment carried out with animals of the M line. However, although a high rate of implantation was obtained in transgenic rats of the M line, the constitutive expression of porcine GH at a mean level of 45 ng/ml in plasma could be sufficiently elevated to cause increased loss of fetuses in late pregnancy. The high absorption after implantation and number of dead pups born in transgenic group supports this contention. Similar findings were obtained both by administration of high doses of GH to pregnant rats (Jørgensen *et al.*, 1991) and with transgenic mice expressing foreign GH (Naar *et al.*, 1991). With the observation in transgenic female mice of MT/hGH•V, PEPCK/bGH and MT/bGH lines, Naar *et al.* (1991) indicated that on day 14 of pregnancy, pregnant transgenic females had significantly more fetuses (live plus dead) than the control group. On the day of birth, transgenic females from each of the three lines had significantly greater percentages of dead pups than did the control group. The reasons for increased fetal mortality in transgenic rats in the present study are presently unknown, and warrants further studies.

At 12 week of age the mean absolute and relative weights of uterus and ovary in transgenic and transgenic zinc-treated female rats of the D line were the same as those of their non-transgenic littermates, which suggests that the increased ovulation in transgenic female rats was not associated with increased ovary size.

At 30 weeks of age the absolute weights of uterus and ovary of the D line in zinc-treated transgenic females were significantly increased

compared to non zinc-treated transgenic and non-transgenic groups, however, the relative weights of uterus and ovary remained the same between the three groups. This suggests that the expression of the fusion gene, constitutively or induced at a certain age in the rat, can enhance the growth of internal organs (see chapter 4. 4.8) including uterus and ovary without impairing reproductive functions in female rats.

Concerning the impact of the GH transgenes on pituitary function, it has been reported that the pituitaries of transgenic mice expressing human GH had reduced numbers of somatotrophs (as low as 1%) which are responsible for the secretion of GH (Palmiter *et al.*, 1983). In the current study animals from the D line had similar relative pituitary weights in transgenic zinc-treated, transgenic and non-transgenic control groups at 12 and 30 weeks of age. However at 30 weeks of age , the absolute weight of pituitary in transgenic zinc-treated group was significantly increased. This suggests that transgene expression sufficient to increase the growth of body and internal organs can do so without affecting the function of pituitary. However, in rats of the M line the mean absolute and relative weights of pituitary at 12 weeks of age were significantly decreased in transgenic group compared to non-transgenic controls, whilst at 30 weeks of age, the relative weight of pituitary was lower although the absolute weight was comparable to that in non-transgenic group. This finding suggests that the function of the pituitary is somehow affected by the constitutive over-production of porcine GH, which may contribute to the delay of puberty in transgenic females of this line.

Regardless of the mechanism involved, it is clear from the present data that expression of porcine GH transgene in transgenic rats

enhanced, rather than impaired, female fertility. These results are in sharp contrast to the well-documented various degrees of impairment of reproductive function found in transgenic female mice expressing human, porcine and bovine GH genes (Michalska, 1988, Naar *et al.* 1991).

The reasons for sterility in transgenic female mice expressing exogenous GH remain to be determined. When daily s.c. injections of 1 mg progesterone are given, starting on the day females had their second plug, Bartke *et al.* (1988) observed that human GH transgenic female mice became pregnant and the pregnancy was carried to term. Furthermore, following transplantation of pituitaries from normal to transgenic females, eight of the ten transgenic females became pregnant and normal pups were delivered (Bartke *et al.*, 1988). This data is interpreted to suggest that sterility of transgenic female mice may be due to one of two mechanisms: 1) a failure of the corpora lutea to respond to a lactogenic stimulus, 2) a suppression of endogenous prolactin release, which normally follows mating (Barkley, 1982) and is responsible for the maintenance of luteal activity (Bartke, 1973). The mechanisms for sterility in transgenic female mice expressing foreign GH's of different species are possibly not the same because human GH is known to be lactogenic in rodents and thus can interfere with endogenous prolactin release (Bartke *et al.*, 1988) while bovine GH is not (Hartree *et al.*, 1965).

The information from the literature indicates that the transgenic female mice used in fertility studies were normally expressing foreign GH constitutively at a high level and grew much faster than normal. It is generally accepted that superabundance of GH can constitute a

strong, non-specific stress and create dramatic physiological changes, which may be interfering with the mechanisms regulating fertility in female animals. Furthermore, breeding lines of transgenic animals, established by microinjection of DNA into pronuclear of zygotes, are all unique depending on the level and the site of expression of the transgene. It can be assumed that expression of the transgene at different levels and sites may affect the reproductive function of female mice in different ways. So, a precise selection of transgenic animal used as a breeding line is possibly a critical step, not only to overcome the sterility in transgenic female mice expressing GH fusion genes, but also to study the action of GH in reproductive functions. Unfortunately, no such work has been done yet in previous studies of transgenesis. The enhanced fertility obtained from the female transgenic rats of both the D and M lines established in the present study, including increased numbers of ovulations, implantations and litter size at birth and weaning, strongly suggests that expression of porcine GH does not necessarily suppress endogenous prolactin release and corpora lutea can retain normal function in transgenic female rats.

In this study a great attention was given to the selection of the animal lines, with a special focus on fertility. The two animal lines selected from 39 founder transgenic rats were all fertile. Following an investigation of seven generations of animals in the M line and five generations of animals in the D line, it is clear that unimpaired fertility together with the integration and expression of the fusion gene were heritable, which firmly confirmed that these animal lines were a suitable model for a fertility study of transgenic female animals expressing GH fusion gene.

In conclusion, expression of porcine GH fusion gene in transgenic rats enhanced, rather than impaired female fertility, a consequence of an enhanced ovulation rate. Both immature and mature female rats expressing porcine GH ovulated more ova than non-transgenic controls, confirming that this level (20 ng/ml) of expression results in some stimulating effect on follicle growth to induce more follicles to undergo continued growth up to ovulation. GH may thus act to amplify the action of the gonadotropins. Constitutive expression of porcine GH at the level of about 40 ng/ml also appeared to increase the milk yield of lactating transgenic female rats, resulting in faster growth of pups during lactation and a higher number of viable pups at weaning, thus demonstrating for the first time that transgenic females expressing foreign GH can not only be fully fertile but also have an enhanced maternal capacity. Although the body weight of transgenic female rats at 30 week of age was increased, when the expression was increased by zinc water, the females had same mating period and oestrous cycles as non-transgenic controls did, and the weights of uterus, ovary and pituitary in relation to body weight were the same as that at 12 weeks of age. Taken together, the information suggests that the fertility potential of transgenic female rats is not compromised when circulating levels of porcine GH are between 20-45 ng/ml.

Table 5.1 The timing of puberty onset of MTIIa/pGH transgenic rats vs non-transgenic littermates in relation to body weight and plasma pGH level.

Genotype	Body Weight (g)	pGH (ng/ml)	Time (days)
Transgenic n=22	122.5±2.1***	42±2	36.2±0.66*
Non-transgenic n=24	97.3±2.7	<8	34.4±0.5

Values are mean ± s.e.m.

* **P<0.05**.

*** **P<0.001**.

Table 5.2 Comparison of the mating pattern of transgenic vs nontransgenic rats

Mating days	Transgenics	Non-transgenics
1		
2		
3	2	2
4	2	2
5		1
6		1
7		1
8		
9	2	1
10	2	
Mean ± s.e.m.	6.5 ± 1.23	5.1 ± 0.8

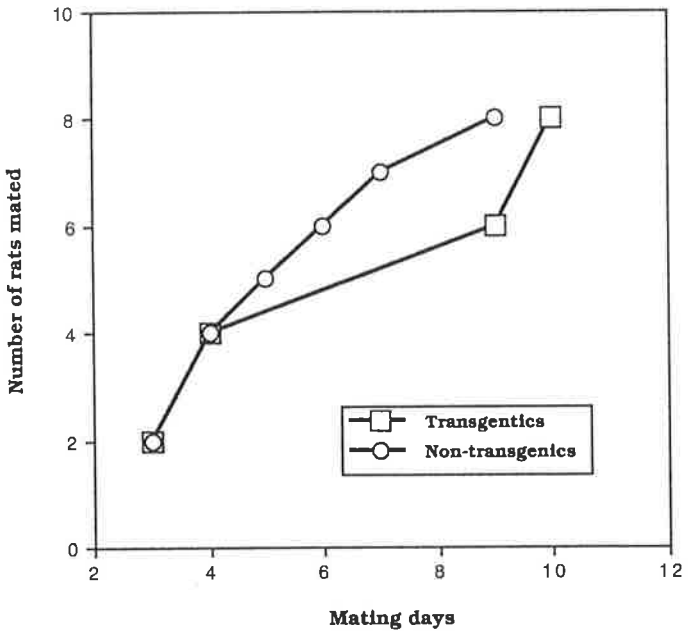


Figure 5.1 Cumulated number of rats mated

Table 5.3 Mean implantation rate, litter size and number of viable pups at birth and at weaning in MTIIa/pGH transgenic and non-transgenic female rats (M line)

Genotype	pGH (ng/ml)	Implantations	Litter size (dead plus live)	Viable pups at birth	Pups at weaning
Transgenic n=8	40±3.6	15.3±0.35**	13.5±0.53	12.8±0.80	11.9±0.56
Non-trans n=8	<8	12.7±0.82	12.4±0.77	12.4±0.77	11.4±0.45

Values are mean ± s.e.m.

** **p<0.01**.

Table 5.4 Mean milk yield in lactating rats (transgenic vs non-transgenic littermates) on days 7 and 10 lactation in relation to litter weight at day 21.

Genotype	Milk at day 7 (g)	Milk at day 10 (g)	Litter weight at day 21 (g)
Transgenic n=8	2.68±0.73	4.68±1*	499.9±10.9*
Non-transgenic n=8	2.03±0.39	2.28±0.48	444.1±16.1

Values are mean ± s.e.m.

* **P<0.05**.

Table 5.5 Live litter weights (LW) of pups weaned from transgenic vs non-transgenic mothers at day 7, 10 and 21 postpartum

Genotype	Birth weight (g)	LW at day 7 (g)	LW at day 10 (g)	LW at day 21 (g)
Transgenics	66.1 ± 0.48	159.7 ± 8.28	224.3 ± 6.0	499.9 ± 10.9 *
Non-transgenics	66.60 ± 0.72	168.3 ± 13.5	229.0 ± 7.5	444.0 ± 16.1

Values are mean ± s.e.m. * P<0.05

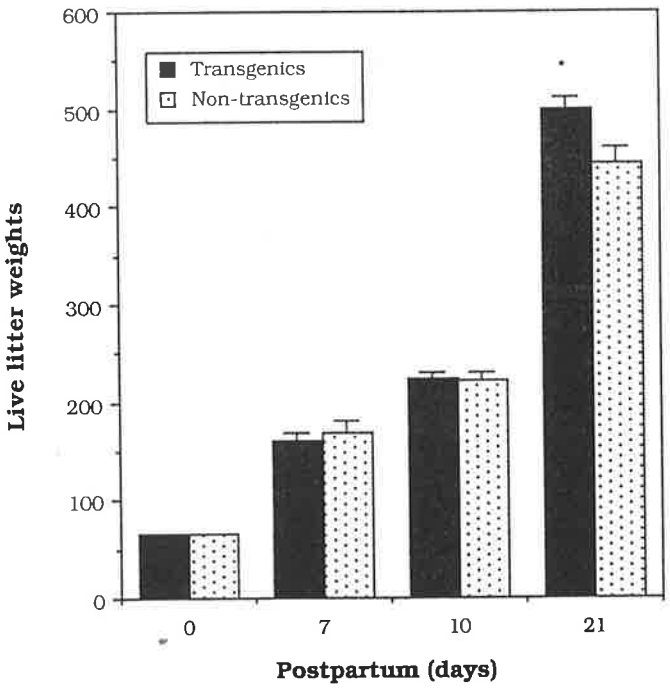


Figure 5.2 Live litter weights of pups weaned from transgenic vs non-trangenic mothers.
*** P<0.05**

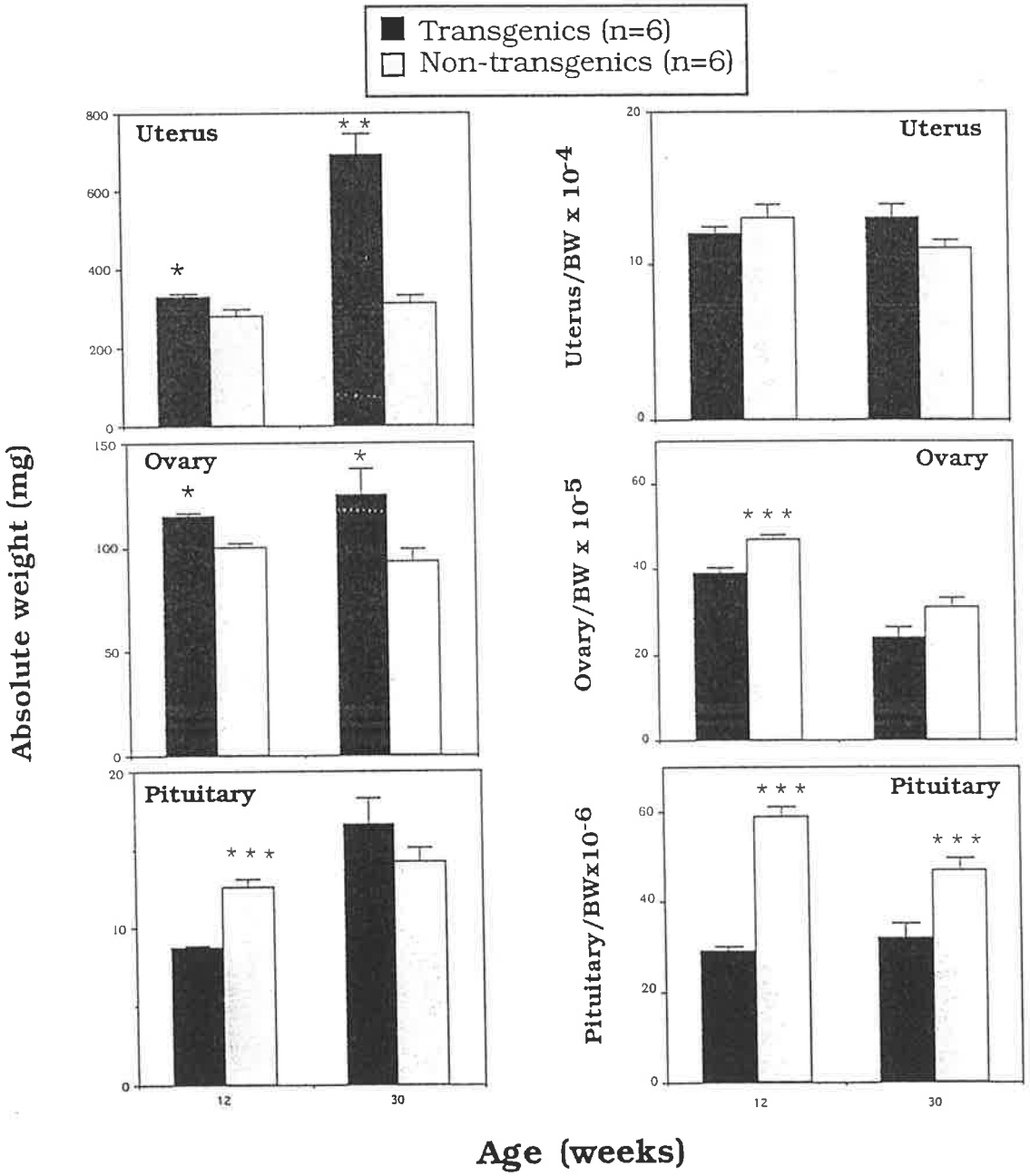


Figure 5.3 The absolute and relative weights (organ/body weight) of uterus, ovary and pituitary of transgenic female rats vs non-transgenic littermates of the M line at 12 and 30 weeks of age.
*** p<0.05; ** p<0.01; *** p<0.001.**

Table 5.6 The effect of chronic pGH secretion on ovulation rate

Genotype	No. rats	pGH (ng/ml)	Oocytes recovered
Transgenic	1	17	34
	2	18	35
	3	16	40
	4	15	25
	5	19	28
	6	23	41
	7	18	38
	8	24	32
	9	15	38
mean ± s.e.m.		**18±1**	**34.6±1.9*** **
Non-transgenic	1	<8	28
	2	<8	25
	3	<8	25
	4	<8	26
	5	<8	19
	6	<8	19
	7	<8	26
	8	<8	30
	9	<8	24
mean ± s.e.m.			**24.7±1.3**

values are mean ± s.e.m..

*** **p<0.001.**

Table 5.7 Number of transgenic (T) vs non-transgenic (NT) femal rats mated per day in the mating period

Mating days	T + Zn^{++}	NT + Zn^{++}	T – Zn^{++}	NT – Zn^{++}
1	2	1		2
2	2	4	3	3
3	3	2	2	1
4	1		1	
5			2	
6				
7		1		2
Mean ± s.e.m.	**2.4 ± 0.4**	**2.6 ± 0.6**	**3.3 ± 0.5**	**3.1 ± 0.9**

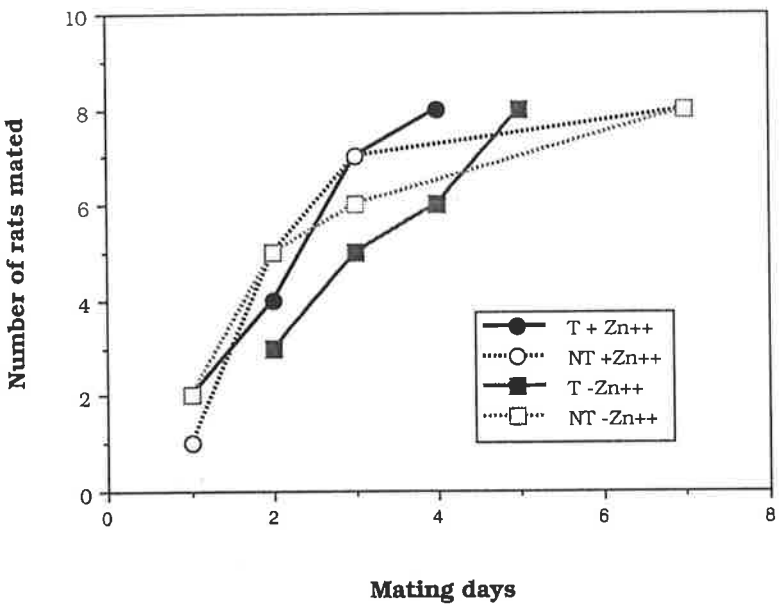


Figure 5.4 Cumulated number of rats mated

Table 5.8 Implantation rates, litter size at birth and weaning of transgenic(T) vs non-transgenic (NT) female rats in relation to plasma pGH and Zn++ exposure

Genotype	Birth weight (g)	treatment (Zn++)	pGH (ng/ml)	implantations*	litter size	pups at weaning
T n=8	5.4±0.09	-	20 ± 3.4	16.8 ± 1.06A	14.6 ± 1.2	11 ± 0.5B
T n=8	5.6±0.05	+	41 ± 3.3	15.5 ± 0.9C	15.3 ± 0.9A	13.8 ± 0.5A
NT n=8	5.6±0.13	-	<8	13.4 ± 0.81BD	12.6 ± 0.8B	9.5 ± 0.8B
NT n=8	5.7±0.09	+	<8	13.5 ± 1.12B	12.9 ± 1.02B	11.1 ± 1.1B

* Uterus implantation scars.

Values are mean ± s.e.m.

A v B P<0.05, C v D P<0.05.

Table 5.9 Implantation rates, litter size and number of pups weaned for transgenic (T) female vs non-transgenic (NT) female rats.

Genotype	Implantation	Litter size	Pups at weaning
T	16.2±0.69**	14.9±0.72*	12.4±0.5*
n=16			
NT	13.4±0.65	12.8±0.59	10.3±0.68
n=16			

Values are mean ± s.e.m.

*** P<0.05**

**** P<0.01.**

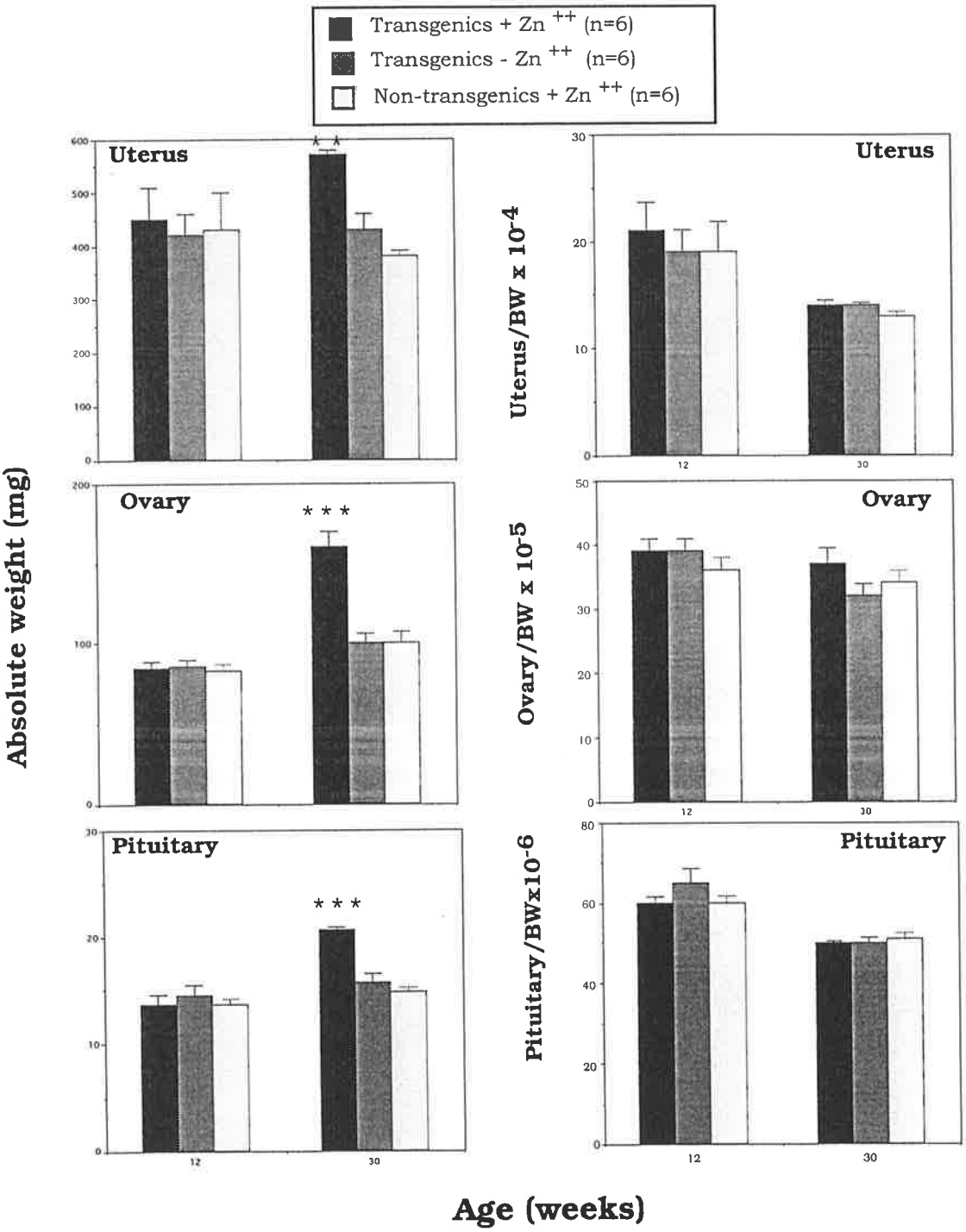


Figure 5.5 The absolute and relative weights (organ/body weight) of uterus, ovary and pituitary of transgenic female rats vs non-transgenic littermates of the D line at 12 and 30 weeks of age.
**** p<0.01; ***p<0.001.**

Chapter6
General discussion

GENERAL DISCUSSION

Since the commencement of this study, several lines of transgenic rats have been established which are proving useful as animal models for studies of hypertension (Mullins *et al.*, 1990), spontaneous inflammatory disease (Hammer *et al.*, 1990) and production of bovine alpha-lactalbumin (bαLA, Hochi *et al.*, 1992). The development of a rat model for growth studies using a transgene comprising hMTIIa promoter linked to a pGH cDNA was initiated to complement studies of the same transgene in transgenic mice and pigs (Michalska, 1988; Vize *et al.*, 1988). Consistent with the success in these species, a high integration frequency of 56% was achieved for the pGH transgene in rats following microinjection. Furthermore, a large proportion of the transgene positive pups (approximately 28%) were found to have immunoreactive pGH in their blood at weaning, at levels which varied from as low as 18 ng/ml to as high as 1200 ng/ml. This variability in level of expression was unrelated to the number of copies of the integrated transgene.

Two stable breeding lines of transgenic rats (Lines M and D) were established following selection from the 11 founder animals, on the basis of inheritance of integration and expression of the transgene, and maintained by outbreeding to unrelated non-transgenic normal rats. Investigation of 7 generations in line M and 5 generations in line D indicated that the integration and expression of the transgene were permanently inheritable in these animal lines, with the expected Mendelian ratio. An investigation of the tissue site of expression of pGH mRNA using a ribonuclease protection assay, showed activity in a number of tissues including the liver, kidney, spleen, heart, intestine,

muscle, uterus and ovary, consistent with the housekeeping role of the metallothionein promoter in its corresponding native gene. The pattern, tissue distribution and abundance of pGH mRNA varied between the two animal lines. In the M line the major site of expression of pGH was in the liver whereas in the D line, greater pGH mRNA was found in the intestine and spleen rather than in the liver. These findings are in general agreement with the results of transgenesis using the MT-I and MT-II promoters in transgenic mice (Palmiter *et al.*, 1986) and support the view that the level of expression of a transgene is heavily influenced by its site of integration within the genome. From studies with mice and other species, it may be assumed that the site of integration differs between Line D and M, but confirmation using chromosomal *in situ* hybridisation (Lacy *et al.*, 1983) which would be feasible using the hMTIIa promoter Hind III/Ava I probe has yet to be made.

Interestingly, pGH mRNA analysis indicated that the transgene was also highly expressed in the uterus and ovary of the M line (see chapter 4.4.5 and figure 4.8), which was firstly identified in GH transgenic animals. At this stage the effect of this high expression of the transgene in the reproductive organs on female fertility remains to be determined. However, a strong recommendation of further study of the relationship between the high expression of the transgene in reproductive organs and female fertility can be made, which may give a better understanding of the effect of overproduction of GH on female fertility of transgenic animals.

Recently Palmiter *et al.*, (1993) have identified DNA regions far upstream and downstream of the mouse MT-I and MT-II genes respectively (DNase I-hypersensitive sites flanking the MT genes, about 6 kb 5' of the MT-II gene and 4 kb 3' of the MT-I gene), which can confer position-independent expression on a variety of reporter constructs in transgenic mice. These regions also increased the *in vivo* expression of intronless copies of the rGH gene. The sequences corresponded to the sites of DNAse hypersensitivity and are believed to function as 'Locus Control' regions for the metallothionein genes. Thus, inclusion of such regions in any future MTIIa/pGH constructs may enable consistent expression of the construct in transgenic rats.

These two lines of transgenic rats show marked differences in both the levels of circulating pGH and their growth response. In the M line, transgenic animals exhibit high levels of plasma pGH (45 ng/ml) and accelerated growth which is noticeable as early as 23 days of age. As the animals grew older, the differences in body size between transgenic and non-transgenic control animals became more pronounced, and in the female rat body weight was 40% greater at 12 weeks and 70% greater by 30 weeks of age. In contrast transgenic animals in the D line exhibited low levels of plasma pGH and growth in these animals was not significantly different from that of their non-transgenic controls. However, the level of pGH expression in this animal line could be increased by exposure of the transgenic animals to zinc, and this inducibility trait is stably heritable. Following exposure to zinc, the expression of pGH was enhanced, resulting in a 46% increase of mean body weight in transgenic female rats at 30 weeks of age over non-

transgenic controls. Post mortem studies on the rats showed the enhanced growth as distributed proportionately to the internal organs, especially to the growth of pituitary, which suggests that somatotrophs of the transgenic rats in this line is not affected when the levels of circulating GH are increased.

At the present time it is not known whether the transgene derived pGH directly acts on the rat GH receptors or whether enhanced growth occurs as a result of displacement of rGH from circulating GH BP and its subsequent action on GH receptors. Hence, further studies to elucidate the mechanism of growth in the MTIIa/pGH rats will need to carefully examine the levels of endogenous hormones and binding proteins in these rats eg rGH (GHBP), rIGF-1 (IGFBPs) and their possible relationships to the growth observed.

It is well documented that expression of human, bovine and porcine GH genes in transgenic mice was often associated with various degrees of impairment of female reproductive function (Palmiter *et al.*, 1982; Hammer *et al.*, 1984; Yun *et al.*, 1987; Bartke *et al.*, 1988; Naar *et al.*, 1991; Michalska, Ph D thesis, 1988). Unlike transgenic mice, there was no evidence that the MTIIa/pGH transgenic rats had any impairment in the length of oestrous cycle, period of matings or pregnancy rate in either the M and the D line. Indeed, the transgenic rats showed some evidence of increased fertility with immature transgenic females of the D line showing a greater ovulation and implantation rate, compared to that in their non-transgenic controls. This was interpreted as a reflection of expression of pGH enhancing

ovulation rate due to the synergistic effect of GH with FSH on ovaries (JØrgensen & Nowak, 1989). Furthermore, a significantly greater litter size in female rats of the D line and a relatively high number of viable pups in the females of the M line at birth and weaning suggests that the high levels of circulating pGH in female rats did not suppress the surges of endogenous PRL release which is believed necessary for inducing and maintaining luteal function during the first half of gestation in the mouse (Bartke *et al.*, 1988). During lactation significantly higher milk production by transgenic females of the M line compared to that by non-transgenic controls provides additional support for this conclusion. The possible importance of zinc in enhancing the survival of pups from birth to weaning remains to be evaluated.

It has been reported that MT-hGH and bGH transgenic mice showed a markedly reduced life-span in comparison to non-transgenic control animals. Most of the animals either had to be sacrificed due to severe clinical symptoms (cachexia) or died spontaneously (Brem *et al.*, 1989; Quaife *et al.*, 1989). During the course of the present study transgenic rats in both lines have shown a similar life-span to non-transgenic littermates with no unusual health or other pathological affections. Thus, given appropriate safeguards, the study indicates that transgenesis can be compatible with health and fertility. Therefore, it should be realistic to anticipate that studies of GH expression in transgenic animals will yield new information on the action of GH. It may become possible to produce selective stimulation of only some targets or functions normally responsive to GH without producing undesirable side effects. It may also become feasible to produce lines of

farm animals with improved growth, feed efficiency, and body composition without concomitant impairments in fertility, endocrine function, carbohydrate metabolism and viability.

Chapter 7
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