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The effect of overexpressing prolactin receptors on cell proliferation and milk protein synthesis in a bovine mammary epithelial cell line

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August, 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements of the degree of Master of Science

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Acknowledgements

I would like to express my sincere appreciation to my supervisor, Dr. Xin Zhao, for his advice, and for the time and effort he put into helping me complete this thesis. As well, thanks to Dr. Costas Karatzas for getting me started, for the use of his laboratory and for the references he passed my way. I would also like to thank the Association of Universities and Colleges of Canada for providing me with an Agriculture and Agri-Food Scholarship to complete this project. Thanks also to Rebecca Keystone for helping me get started, for being patient with me and teaching me. As well, thanks to Lori Long for her technical advice and for commiserating with me when things did not work. I would also like to thank Nissab Boudjelab for her patience in teaching me ELISA, for helping me learn to think like a scientist, and for giving me a chance to practice my French. A big thank-you to Jai-Wei Lee for putting together a computer at the last minute so I could work on this thesis from far away. Finally, thanks to Wayne for your patience and support over the last two years – you've been more than wonderful!

Abstract

The effect of overexpressing prolactin receptors on cell proliferation and milk protein synthesis in a bovine mammary epithelial cell line.

The Mac-T cell system was used to investigate the role of the prolactin (PRL) receptor in cell proliferation and the regulation of milk protein synthesis. This study was designed to investigate whether overexpressing the PRLR in the Mac-T cell line resulted in a change in its growth rate and an enhancement of its ability to produce milk proteins. To accomplish these goals, Mac-T cells were stably transfected with the rabbit prolactin receptor gene. Fifteen clones and a pool of transfectants were obtained. Of these, one clone and the pool were positive for the PRL receptor expression. The clone (S15) and pool (SP) cells were sorted into high (H), medium (M), and low (L) expressors of the PRLR. The high expressors were used for all subsequent experiments. The presence of high levels of the PRLR on the surface of S15 and SP cells was further confirmed by receptor binding assay and Western Blot. Following the establishment of these cell lines, the cells were used to investigate the effect of increased levels of PRLR on cell proliferation and milk protein synthesis.

It was found that the growth rate of parental cells was depressed in the presence of 5 μg/ml of PRL. In contrast, the growth rate of the transfectants was enhanced by the addition of 5 μg/ml PRL to the culture medium. In addition, both "SP" and "S15" cells produced higher levels of STAT5 upon long-term (48 h) PRL stimulation. No effect on the synthesis of α_{S1}- and β-caseins was noted. It is likely that no differences in protein synthesis were observed because the cells have lost the ability to differentiate, even when cultured on collagen gels in the presence of lactogenic hormones.

Abstract (French)

L'effet de la surexpression des recepteurs prolactine sur la prolifération et la synthèse des protéines du lait dans une lignée cellulaire épithéliale de la glande mammaire bovine

Le système cellulaire MAC-T a été utilisé pour étudier le rôle des recepteurs prolactine (PRL-R) sur leur prolifération et la régulation de la synthèse des protéines du lait. Cette étude a été établie pour vérifier comment la sur-expression des recepteurs PRL dans la lignée cellualire MAC-T resulterais en un changement de sa croissance et l'augmentation de son abilité à produire des protéines du lait. Afin d'élucider ces objectifs, les MAC-T ont été transfectées avec le gène du R-PRL lapin. Quinze clones et un pool des transfectants ont été retenus. Parmi ces clones, 1 clone et le pool étaient positifs pour l'expression du R-PRL. Le clone S15 et le pool SP ont été classés en fonction de leur niveaux d'expression, a savoir élevé, moyen, ou faible. Les expresseurs élevés ont été utilisés pour les experiementations ulterieures. La présence de niveaux élevés du R-PRL sur la surface des cellules S15 et SP a été confirmée par un essai de fixation du recepteur et par western blot. Suite à l'établissement de ces lignées cellulaires, les cellules ont été utilisées pour investiguer l'effet des niveaux de R-PRL sur la prolifération des cellules et la synthèse des protéines du lait.

Il a été prouvé que la vitesse de croissance des cellules parentales a diminué en présence de 5 μg/ml de PRL. Au contraire, la vitesse des transfectants a été augmentée par l'addition de 5 μg/ml de PRL au milieu de culture. De plus, aussi bien les cellules SP et S15 produisent des niveaux élevés de STAT 5 sur une longue durée (48h) de stimulation avec la PRL. Aucun effet sur la synthèse des caséines αs-1 et β n'a été constaté. Il semblerait qu'aucune différence de la synthèse des protéines n'a été observée

car les cellules ont perdu leur abilité a se différencier même quand elles sont cultivées sur des gels de collagène en présence d'hormones lactogènes.

List of Abbreviations

ACTH	Adrenocorticotropic Hormone
CAT	Chloramphenicol Transferase
CHO	Chinese Hamster Ovary Cells
CN	Casein
COS cells	African Green Monkey Kidney cells
DA	Dopamine
DEX	Dexamethasone
E_2	Estradiol
FIL	Feedback Inhibitor of Lactation
GH	Growth Hormone
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GR	Glucocorticoid Receptor
Grb-2	Growth Factor Receptor Bound Protein
GRE	Glucocorticoid Response Element
IGF-I	Insulin-Like Growth Factor I
IGFBP	Insulin-Like Growth Factor Binding Protein
IL	Interleukin
JAK	Janus Kinase
LH	Luteinizing Hormone
MG	Mammary Gland
MGF	Mammary Gland Factor
Nb2	Rat pre-T lymphoma cell line
PKC	Protein Kinase C
PL	Placental Lactogen
PRE	Prolactin Response Element
PRF	Prolactin Releasing Hormone
PRL	Prolactin
PRLR	Prolactin Receptor
PTP	Protein Tyrosine Phosphatase
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SH2	Src-homology region 2
SHC	Src-Homology region adaptor protein
SOS	Son of Sevenless
STAT	Signal Transducer and Activator of Transcription
UTR	Untranslated Region
VIP	Vasoactive Intestinal Peptide

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Introduction and Literature Review

1. The Mammary Gland

The mammary gland is an organ common to all mammals with its function to supply nutrients to the neonate. This is unique to mammals since there are few other animal groups that provide food for its young in this way. Mammalian neonates have a poorly developed digestive tract and nutrients must be supplied in a manner that is compatible with their immature system. As well as supplying food, milk provides the neonate with some protection from disease in the form of antibodies and other molecules for the first few months of its life. The unique relationship between the mammalian parent and its young, although requiring considerable energy on the part of the mother, enables mammals to survive in a wide variety of environments.

1.1 Bovine Mammary Gland Development

1.1.1 In Utero Development

The mammary gland begins to develop very early in embryogenesis as a primordial thickening of the ventrolateral aspect of the ectoderm called the mammary band (Anderson, 1985). This occurs at about day 32 of embryo development in the cow. Approximately two days later, the band thickens into a distinct line that is termed the mammary streak. A subtle change follows, resulting in the orientation of the epithelial cells along a straight line in a distinct area called the mammary line. Rapid proliferation of cells in certain areas along this line, which eventually forms the teat and gland, signals the formation of the mammary crest by day 37 in the cow. The mammary crest then forms the mammary hillock, a hemisphere of epithelial cells, which grow into and crowds the mesoderm below. Near the end of the embryonic phase of growth, day 43 in the cow, the hillock becomes a mammary bud which will form the structures for milk secretion (Anderson, 1985).

During the fetal period of growth, the mammary bud forms a primary sprout. The primary sprout is a primordial structure that becomes the opening in the teat from which milk exits (the galactophore). In cows there is only one opening per teat. The second phase of mammary development in the fetus is the canalization of the primary sprout. That is, the sprout starts to grow very quickly so that soon it is bigger than the bud itself. The epithelial cells in the centre can no longer be nourished and therefore begin to die off, forming a canal in the centre. This is followed by the development of branches from the primary sprout called secondary sprouts. These will eventually become large milk ducts that lead to the cistern.

Following secondary sprout formation, an early layer of adipose tissue is laid down around the mammary bud, which is the beginning of the mammary fatty pad. The growth of the fatty pad is much more extensive in female young than in males.

1.1.2 Post-Partum Development

Following birth, the growth rate of the mammary gland is the same as that for the rest of the body until the cow reaches puberty. At puberty, follicle stimulating hormone (FSH) and luteinizing hormone (LH) begin to be released cyclically. These hormones stimulate the release of estrogens and progesterone from the ovaries. The surge of estrogen during each cycle acts synergistically with prolactin (PRL) and growth hormone (GH) to stimulate mammary gland cells to proliferate. These hormones cause ducts to lengthen and branching to occur. Following the follicular phase, there is a luteal phase in which progesterone is secreted by the corpus luteum.

1.1.3 Effects of Pregnancy on Mammary Gland Development

Hormonally regulated mammary growth and development occurs during pregnancy. The process of development begins slowly and accelerates as pregnancy progresses. Adipose cells are slowly eroded within the parenchyma and replaced with ducts and lobules of alveoli (Bath *et al.*, 1985). In addition, lymph space, connective tissue, and blood vessels develop in preparation for lactation. In fact, a capillary network and myoepithelial cells surround each alveolus (Bath *et al.*, 1985). Growth of the mammary gland is exponential during pregnancy and continues into early lactation. The amount of milk produced depends on a wide variety of factors including the time of initial breeding and udder weight (Bath *et al.*, 1985), environmental conditions such as temperature, and the number of secreting cells available. Other factors include the availability of precursors for milk components in the blood, hormone levels and the frequency of milk removal (Knight and Wilde, 1993; Anderson, 1985).

1.1.4 Involution of the Bovine Mammary Gland

Ceasing milk removal accelerates the rate of involution, or degeneration, of lobule alveoli. Until recently this has been thought to be due to an increase in pressure in the gland. However, Tolkunov and Markov (1997) showed that extending the time period after the cessation of suckling in mice did not result in an increase in intramammary pressure, suggesting that involution may involve other mechanisms.

Litter removal or PRL and GH deprivation can induce involution in rats (Travers *et al.*, 1996). According to these researchers, involution was stimulated by the accumulation of milk that occurred when pups were removed; lack of PRL and GH resulted in decreasing the numbers of secretory cells through apoptosis, as evidenced by the increase in DNA ladders and decreased DNA content (Travers *et al.*, 1996).

In cattle, the degree to which a mammary gland undergoes involution depends on the reproductive state of the animal (Bath *et al.*, 1985). In a non-pregnant cow the udder fills with milk for a few days following weaning, but the activity of secretory cells in the gland decreases (Anderson, 1985). In some species, such as the mouse, alveolar cells then undergo apoptosis and are lost (Li *et al.*, 1997). However, in the bovine, extensive loss of epithelial cells does not seem to occur during involution (Hurley, 1989). Rather, changes in alveolar function occur, such as increased secretion of certain enzymes, and autophagocytosis (Hurley, 1989).

1.2 Regulation of Mammary Growth and Milk Secretion

The events of mammary growth, development, and secretion, including both lactogenesis and galactopoeisis, are regulated by a complex mechanism involving both neural and endocrinological control. Neither the neural nor endocrine controls have been completely elucidated. But at this point, it seems that the involvement of the nervous system is minor or indirect whereas the endocrine system plays a major and more direct role in mammary gland regulation.

The main part of the brain involved in co-ordinating neural/endocrine interactions is the hypothalamus. The hypothalamus controls the secretion of hormones from the anterior and posterior pituitary, based on the neural and hormonal signals it receives. The anterior pituitary is controlled by the hypothalamus that sends inhibiting or releasing factors through the hypothalamic-pituitary portal vessels, while the posterior pituitary receives direct nervous stimulation from the hypothalamus. The main hormones affecting mammary growth and milk secretion are estrogens, progestins, PRL, GH and placental lactogen (PL) (Erb *et al.*, 1976). Prolactin and GH are released from the anterior pituitary, whereas estrogens and progestins are secreted mainly from the ovaries and PL from the placenta. Along with these hormones there are a number of others that influence lactation at various stages of reproduction such as insulin, adrenocorticotropic hormone (ACTH) and corticosteroids.

1.2.1 Estrogens and Progestins

The estrogen most involved in regulating mammary activities is estradiol (E_2), while the main progestin is progesterone. The anterior pituitary hormones LH and FSH stimulate the release of E_2 and progesterone from the ovaries. Progesterone and E_2 act synergistically to stimulate mammary growth during puberty and pregnancy. Progesterone alone can stimulate alveolar development, but both hormones are needed for proper development during pregnancy. Progesterone and E_2 concentrations in blood plasma vary cyclically: concentrations of E_2 in blood are highest during proestrus and lowest during the luteal phase, whereas concentrations of progesterone have opposite trends. However, when the animal is pregnant, blood E_2 and progesterone concentrations increase together, resulting in sustained growth of the mammary gland. These hormones promote cell division in terminal end buds and along duct walls (Tucker, 1985).

Lactogenesis is the process of differentiation of mammary alveoli, such that the cells become capable of secreting milk (Tucker, 1985). It appears that E_2 has a small role in this process, but the absence of progesterone is necessary for lactogenesis. During pregnancy progesterone blocks α-lactalbumin secretion, a component of the enzyme needed for lactose production (Bath *et al.*, 1985). Just prior to giving birth the mother's serum progesterone concentration drops sharply, stimulating the release of E_2, ACTH, and PRL (Erb *et al.*, 1976). In fact, injection of E_2 or glucocorticoids at this stage stimulates milk production in ruminants (Bath *et al.*, 1985).

1.2.2 Prolactin

Control of PRL Secretion

PRL is synthesized in lactotrophs (PRL producing cells) located in the area of the anterior pituitary gland that is close to the posterior pituitary (Horvath and Kovacs, 1994). These cells produce two main forms of PRL, big and small PRL (Horvath and Kovacs, 1994). Within lactotrophs, small PRL seems to be associated with organelles related to the synthesis and processing of the molecule; "big" PRL, that is, PRL molecules bound together (covalently or through non-covalent interactions) to form polymers (Sinha, 1995), is normally found in secretory granules (Torres and Aoki, 1985). Other structural variants of PRL have been identified, resulting either from alternate splicing of a single mRNA transcript (Miller and Eberhardt, 1983), or from post-translational modifications such as glycosylation, cleavage, phosphorylation, or deamination (Kim and Brooks, 1993; Sinha, 1995; Lamberts and Macleod, 1990).

The main regulator of PRL release is dopamine from the hypothalamus (Ben-Jonathan, 1994). Dopamine acts by inhibiting the release of PRL through a mechanism that is not yet completely understood. The mechanism may involve the formation of a physical barrier to secretion (Joneja *et al.*, 1993; Saunders *et al.*, 1983; Reifel *et al.*, 1983, 1985;) or lysosomal action (Nansel *et al.*, 1981). Other inhibitors of PRL release are GAP (gonadotropin releasing hormone associated peptide), endothelins and calcitonin (Ben-Jonathan, 1994). As well, dopamine released from the posterior pituitary may play an important role in controlling PRL output (Hazerligg *et al.*, 1996; Ben-Jonathan, 1994; Hyde and Ben-Jonathan, 1988, 1989; Murai and Ben-Jonathan, 1987).

PRL release can also be stimulated by releasing factors. Substances known to cause PRL release include thyrotropin releasing hormone (TRH) from the hypothalamus (Hoyt and Tashjuan, 1980; Nakajima *et al.*, 1993), and vasoactive intestinal peptide (VIP), which originates both from the hypothalamus and from lactotrophs themselves (Ben-Jonathan, 1994; Nagy *et al.* 1988). Another compound that is released from the posterior pituitary seems to be a much more potent PRL releasing factor (PRF; Hazerligg *et al.*, 1996; Hyde and Ben-Jonathan, 1988, 1989; Murai and Ben-Jonathan, 1987). This putative PRF has not yet been characterized.

Factors released from remote areas of the body, such as serotonin, opioids and estrogen, can also increase plasma concentrations of PRL (Ben-Jonathan, 1994). In addition, prolonged litter removal in rats (20 hours or longer) resulted in an increase in plasma PRL (Yamamuro and Sensui, 1997). They hypothesized that this was due to a transfer of PRL from the mammary gland (that is, from the milk produced since the last feeding) into blood plasma since a concurrent decrease in milk PRL was noted.

PRL function

The major function of PRL in many mammals is the growth of the mammary gland, and the initiation and maintenance of lactation during and subsequent to pregnancy (Vonderhaar, 1987; Ben-Jonathan, 1985). However, PRL has many other diverse effects in mammals besides its stimulatory effect on lactation. These include regulating growth, differentiation, gonadal development and function, skin and hair growth, the immune response (Jabbour and Kelly, 1997) and regulating the synthesis of other reproductive hormones (Martel *et al.*, 1990).

Despite the diversity of its effects, the main function of PRL in mammals is believed to be the regulation of normal mammary gland growth, differentiation, and function. In adrenalectomized-hypophysectomized rodents it has been found that E_2 and adrenal steroids, plus either GH or PRL, are necessary for mammary ductal growth (Ormandy *et al.*, 1993). As well, E_2, progesterone and PRL are necessary for normal lobuloalveolar development (Ormandy *et al.*, 1993). In this case, the effects of E_2 were thought to be indirect, and it was suggested that E_2 stimulated the mammary gland to develop by inducing PRL release (Sheth *et al.*, 1978, Hayden, 1979). *In vitro*, if PRL was removed from the medium of cultured, differentiated cells, involution occurred (Ormandy *et al.*, 1993). Additionally, PRL acts as a major mammary tumour mitogen in rodents (Ormandy *et al.*. 1993). Interestingly, this function of PRL is not noted in the bovine. Collier *et al.* (1993) showed that, both *in vivo* (by blocking PRL secretion) and *in vitro* (using cultured bovine mammary epithelial cells from pregnant non-lactating heifers), PRL did not act directly as a mitogen for the bovine mammary gland. As well, they showed that PRL did not act locally nor systemically *in vivo*.

For many species PRL initiates and maintains lactation; in dairy cattle and some other ruminants the role of PRL is not as well understood. It is known that PRL is necessary for lactogenesis in ruminants (Bath *et al.*, 1985; Akers *et al.*, 1981). Akers *et al.* showed a 45% decrease in initial milk yield in cows treated with an inhibitor of PRL release for 12 days prior to parturition. Interestingly, milk yield increased to near control levels as lactation progressed (Akers *et al.*, 1981).

However, the role PRL plays in galactopoesis is not clear in the bovine. Milking induced a hormonal response in ruminants (that is, PRL, ACTH, and OT release), but PRL responded only briefly (less than 30 min) and its effect on the mammary gland is unknown (Bath *et al.*, 1985). In addition, cows treated with a PRL inhibitor did not show a decrease in milk yield in established lactation (Yanai and Nagasawa, 1974; Fell *et al.*, 1974). Plaut *et al.* (1987) found that increased plasma PRL during early (days 21-34 postpartum) and after peak (days 60-73 postpartum) lactation had no effect on either milk yield or milk components (fat, lactose and protein) during those periods. From these studies it is evident that PRL plays little role in maintaining milk production in established lactation in the cow.

1.2.3 Other Hormones Involved in Mammary Growth and Milk Secretion

Placental Lactogen

Placental lactogen is similar in chemical properties and composition to PRL and GH, although it is not a glycoprotein (Anderson, 1985). PL is a 25-35 kDa protein which synergizes with estrogen and progesterone to stimulate growth in the mammary gland (Anderson, 1985). During early pregnancy maternal hormones play the most important role in the development of the mammary gland, but at mid-pregnancy the placenta becomes a much more significant producer of lactogenic hormones such as PL (Tucker, 1985). In many species, this secretion is maintained from mid-pregnancy to parturition. However, it is not known to be involved in the regulation of milk secretion in ruminants (Byatt *et al.*, 1997; Tchelet *et al.*, 1995; Byatt and Bremel, 1986), although it has been shown to be mitogenic in cows (Collier *et al.*, 1993).

Growth Hormone

The ability of GH to increase milk production in livestock has been known for 60 years (Bauman and Vernon, 1993; McDowell, 1991). However, widespread use and study of GH has been limited, until recently, by the limited availability of pituitary derived GH. With the advent of recombinant technology, recombinant bovine GH (rbGH) has become readily available, resulting in a surge of research and use of the hormone (Bauman and Vernon, 1993; McDowell, 1991). rbGH is now widely used in some countries to increase milk production although the mechanisms by which this takes place are largely speculative. However, it is known that stimulation with rbGH does not change the composition of milk neither *in vivo* nor *in vitro* (Barbano *et al.*, 1992; Laurent *et al.*, 1992; Peel and Bauman, 1987) suggesting that rbGH does stimulate component synthesis, but all are equally increased.

The mechanisms by which milk yield is increased after rbGH injection are mainly thought to be indirect. Some of the effects seen following rbGH injection involve changes in the animal's partitioning of absorbed nutrients (Breier *et al.*, 1991). That is, lipid accumulation is decreased, and fatty acids are used as energy; other nutrients in feed can then be used to produce milk (Breier *et al.*, 1991). Researchers have also noted an increase in the uptake of nutrients by the mammary gland, increased milk synthesis, increased activity of individual secretory cells in the mammary gland, and increased blood flow to the mammary gland in animals treated with rbGH (Bauman and Vernon, 1993; Breier *et al.*, 1991). Whether the number of secretory cells is increased by rbGH seems to be in question, although one reviewer suggests that this is unlikely, especially during late pregnancy (McDowell, 1991). McDowell (1991) also suggests that some of the effects of rbGH may be due to its ability to increase plasma IGF-I concentrations. There are a number of facts that may support this hypothesis. First, IGF-I receptors are detectable in the bovine mammary gland (Cohick, 1998). Second, IGF-I has a much longer half-life in the circulation than does GH (Gourmelen *et al.*, 1994) due in part to the binding of IGF to carrier proteins in the blood. Third, studies have suggested that IGF-I may act as a vasodilator, facilitating an increased blood flow to the mammary gland (Glimm *et al.*, 1988).

GH-releasing hormone (GHRH) and GH regulate the release of endogenous bGH from the anterior pituitary. The actions of both endogenous and rbGH are mediated by the GH receptor (GHR) and have similar potencies (Bauman and Vernon, 1993). The cDNA for the GHR in many species codes for a 620 aa protein (Bauman and Vernon, 1993) which migrates at either 67 kDa (Haeuptle *et al.*, 1983) or 120-130 kDa (Carter-Su, 1996) on SDS polyacrylamide gel. The discrepancy between these two figures may be accounted for by different post-translational modifications of the receptors. The study by Haeuptle *et al.* (1983) showed two GH binding sites in rabbit liver: a 67 and a 35 kDa forms. They suggested that the 35 kDa form mediates the lactogenic actions of GH because it alone was detected in the mammary gland.

The GHR shares many of the common features of cytokine receptors, including Box-1 and Box-2 in the cytoplasmic region (Carter-Su *et al.*, 1996). The highest numbers of both PRLR and GHR are found in the liver of many species, including the rabbit, rat, sheep, and pigeon (Posner *et al.*, 1974); pregnancy increases binding in the liver of rats and rabbits. Pregnant rabbits also show high levels (>3% binding) of GH binding in the mammary gland, ovaries and adrenals (Posner *et al.*, 1974). However, there were no detectable GHR in the bovine mammary gland (Gertler *et al.*, 1984), although GHR mRNA has been detected in the alveolar epithelial cell region (Glimm *et al.*, 1990). In the mammary glands of rabbits in late gestation or early lactation, binding occurred via another receptor type, which has been showed to be a 35 kDa protein (Haeuptle *et al.*, 1983).

Feedback Inhibitor of Lactation

The possibility that a protein in milk regulates milk synthesis was first proposed by Linzell and Peaker (1971). With the knowledge available at that time, they suggested that the action of PRL on mammary secretory cells might be mediated by a component of milk such as calcium, magnesium, citrate, phosphate, lactose, or fatty acids. However, recent studies have shown the presence of a novel protein that has been demonstrated to be inhibitory to the secretion of milk components.

Wilde *et al.* (1995a) purified the protein responsible for inhibiting milk synthesis in goats. They identified a 7600 Da whey protein that has no homology to any other milk protein or any other known protein; evidence for a similar protein exists for human, mice, sheep, and other species (Kim *et al.*, 1997; Prentice *et al.*, 1988; Wilde *et al.*, 1987). This protein has been termed the feedback inhibitor of lactation (FIL). Wilde *et al.* (1995a) also demonstrated *in vitro* that FIL was secreted by mammary epithelial cells into the alveolar lumen, along with other milk proteins. The fact that FIL operates in a concentration dependent manner suggests a mechanism by which the concentration of FIL in milk increases as milk accumulates, slowing milk production via a negative feedback mechanism. Other studies showing that more frequent milking resulted in an increase in milk yield agree with this theory (Wilde *et al.*, 1995b; Stelwagen *et al.*, 1996; Knight *et al.*, 1990; Henderson and Peaker, 1984).

The FIL has been shown to decrease the number of PRLR on mammary epithelial cells in the goat (Bennett *et al.*, 1990; McKinnon *et al.*, 1988), suggesting that one of the mechanisms by which FIL decreases milk secretion is by decreasing the sensitivity of mammary epithelial cells to stimulation by PRL (Bennett *et al.*, 1990).

2. The Growth Hormone/Prolactin Receptor Family

2.1 Introduction

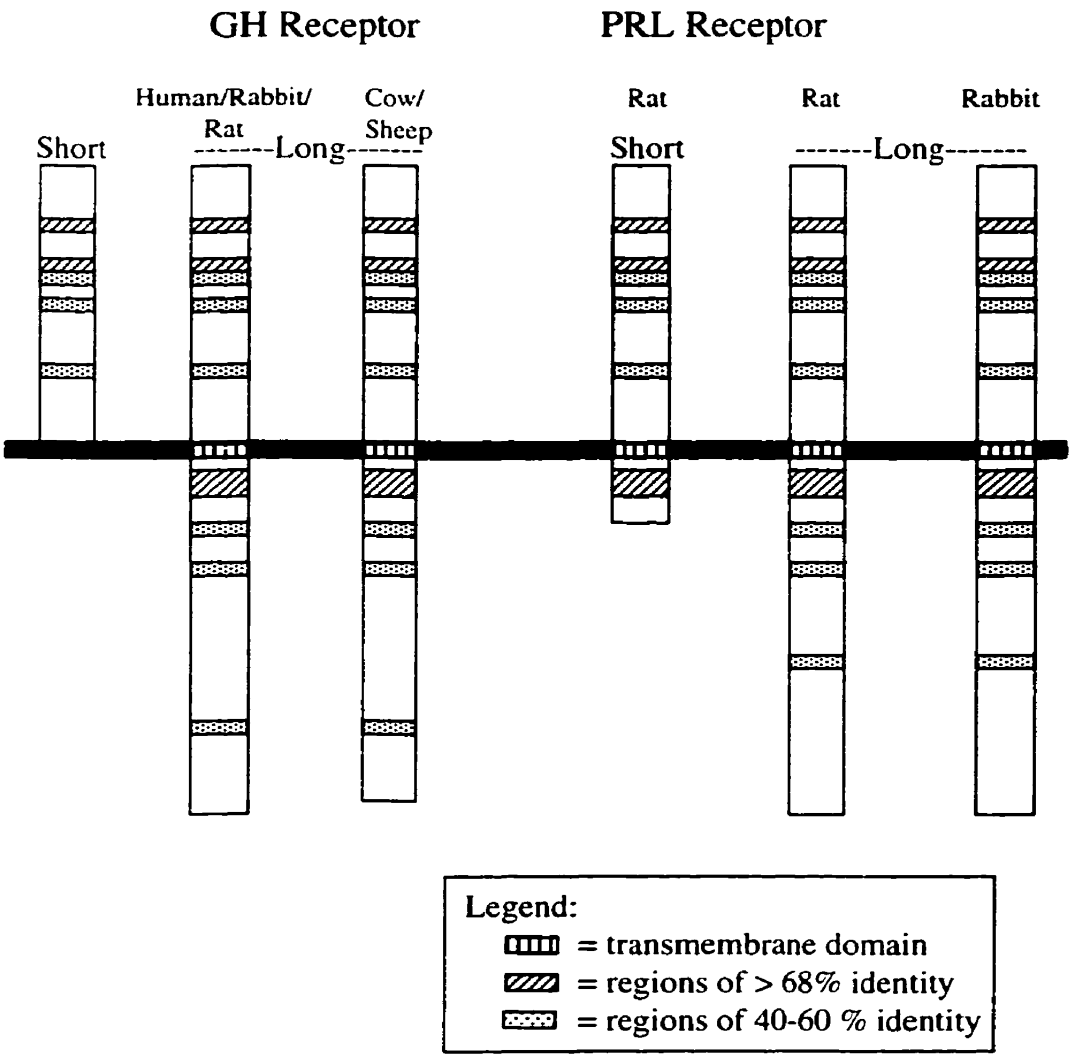
The growth hormone and prolactin receptor family is part of a larger family of receptors known as the haematopoietic or cytokine receptor superfamily. This superfamily includes receptors for lymphokines, monokines, growth factors, polypeptide hormones and other polypeptide factors (Cosman, 1993: Bazan, 1989). All of these receptors exist in more than one molecular form, but as a group have a number of characteristic features. All receptors in this family have three domains: a single transmembrane domain, an extracellular ligand binding domain, and an intracellular domain (Gourdou *et al.*, 1996). No intrinsic tyrosine or threonine kinase activity has been reported for any member of this group (Gourdou *et al.*, 1996).

Members of the cytokine receptor superfamily have low overall structural homology, varying between 14-25% (Kelly *et al.*, 1993). Both GH and PRL have selected regions of strong homology with PL, and together make up a family of polypeptide lactogenic hormones (Kelly *et al.*, 1993). Correspondingly, the receptors for GH and PRL exhibit a fairly high degree of structural (Figure 1) and sequence homology. As well, there is an overall sequence identity of about 30% between the two receptors, which in some areas can be as high as 70% (Kelly *et al.*, 1993; Waters *et al.*, 1990). GH can bind to both the GHR and the PRLR; binding is enhanced by the addition of Zn^+ (Cunningham *et al.*, 1990).

The ligand binding domains of cytokine receptors encompass approximately 200 aa (Gourdou *et al.*, 1996). This 200 aa region can be subdivided into two domains of approximately 100 aa each (Cosman, 1993). Within these conserved regions of the ligand binding domain, there are three defining characteristics among hematopoetic receptors: 1. The WSXWS (Try-Ser-N-Try-Ser) box near the membrane in the extracellular domain (Gourdou *et al.*, 1996). This sequence seems to be important for regulating ligand binding (Rozakis-Adcock and Kelly, 1991) and is present in all members of this superfamily except the GH receptor (Kelly *et al.*, 1993); 2. Four conserved cysteine residues located in the amino terminal half of the extracellular domain. These cysteine residues have been shown to form disulfide bonds in the GH receptor (Fuh *et al.*, 1993) and may be an integral part of the formation of ligand binding pockets (Kelly *et al.*, 1993); 3. Two conserved motifs within the cytoplasmic domain called Box 1 and Box 2. Box 1 has a proline-rich region which is known to be involved with protein-protein interactions (Ren *et al.*, 1993) and is important for signal transduction in the human GHR (Colosi *et al.*, 1993). In particular, the proline in the last position seems to be very important for proper functioning of the receptor (Pezet *et al.*, 1997). Box 2 has no known consensus sequences or function (Lebrun *et al.*, 1995b).

In addition to these three characteristics, some members of the family also have fibronectin III-like modules (Cosman, 1993; Patthy, 1990) or immunoglobin-like domains in the extracellular domain (Cosman, 1993). When present, it is likely that the fibronectin-like modules are important in ligand binding (Somers *et al.*, 1994).

Figure 1: Schematic representation of the GH/PRL receptor family. The long and short forms of the GH receptor in rabbit, cow, sheep, and rat are compared to the short form of the PRLR in the rat, and the long form of the PRLR in the rat and rabbit. Adapted from Kelly *et al.*, 1993.


GH Receptor
PRL Receptor
Human/Rabbit/ Rat
Cow/ Sheep
Rat
Rat
Rabbit
Short
-------Long-------
Short
-------Long-------
Legend:
▥ = transmembrane domain
▨ = regions of > 68% identity
░ = regions of 40-60 % identity

2.1.1 The Growth Hormone/Growth Hormone Receptor System

The GH/GHR system is widely accepted as the model system for members of the cytokine family of receptors (Cosman, 1993). The structure of the human GHR was elucidated by X-ray crystallography. This revealed that the two 100 aa domains of the ligand binding fold into seven anti-parallel ß-strands (Thoreau *et al.*, 1991; Bazan, 1990a) which form a sandwich of 2 ß-sheets (Cosman, 1993). The two domains are held together by a short, four aa linker sequence (Cosman, 1993). The binding of GH to its receptor has also been studied, showing that one molecule of hormone binds with two receptor molecules at essentially the same region on the receptor (Cosman, 1993).

2.1.2 Forms of Cytokine Receptors

Both the GHR and the PRLR exist in multiple forms (Kelly *et al.*, 1993), although both are coded for by single genes. There is evidence that some of the receptors in the haematopoetic family, such as the GHR (Sadeghi *et al.*, 1990) and the PRLR (Postel-Vinay *et al.*, 1991; Mercado and Baumann, 1994), exist in soluble forms (Cosman, 1993). This phenomenon results from either alternate mRNA splicing, which results in the splicing out of the transmembrane region and membrane proximal charged residues (Cosman, 1993), or from proteolysis at the cell membrane, as shown for the GHR (Sadeghi *et al.*, 1990). The function of these soluble receptors is not known, but they may act as cytokine antagonists. For example, a soluble form of the PRLR could down-regulate the function of PRL by binding it before it reaches membrane bound forms of the receptor (Jabbour and Kelly, 1997). Another possibility is that soluble forms act as carrier proteins for cytokines in the blood (Cosman, 1993). Alternatively, they could be non-functional, a hypothesis which is supported by the very low levels of mRNA detectable for soluble forms of most cytokine receptors (Cosman, 1993). A soluble form of the PRLR in milk is suggested to act as a transporter molecule for the delivery of PRL to the infant intestine (Jabbour and Kelly. 1997).

2.1.3 Cytokine Signal Transduction

Although no cytokine receptor exhibits intrinsic catalytic activity, all members of this receptor superfamily are associated with tyrosine kinase transducers, such as members of the Janus Kinase (JAK) family (Gourdou *et al.*, 1996). For example, signalling by the PRL (Campbell *et al.*, 1994: Lebrun *et al.*, 1994; Rui *et al.*, 1994), GH (Argetsinger *et al.*, 1993). erythropoietin (Witthuhn *et al.*, 1993), and IL-3 (Silvennoinen *et al.*, 1993) receptors involves JAK2 kinase. Some of these receptors, such as the PRLR, are constitutively associated with JAK2 (Goupille *et al.*, 1997; Lebrun *et al.*, 1994). There are four known JAKs. JAK1. JAK2, JAK3. and TYK2. JAK2 has been shown to be activated by many cytokines, including IL-3. IL-5, GH, and GM-CSF (Gao *et al.*, 1996; Ferrag *et al.*, 1996). JAK1 is involved with the signalling of the receptors for IL-1 to IL-7. JAK3 is expressed in T-cells, myloid cells, and breast tissue derived cell lines (Witthuhn *et al.*, 1994).

Other substrates besides JAK proteins are tyrosine phosphorylated when cytokine receptors are activated, such as phosphoinositol-3 kinase (PI-3 kinase; Remillard *et al.*, 1991) or Raf-1 kinase (Miyajima *et al.*, 1992). Many of the events that follow receptor activation are the same as those resulting from signalling by tyrosine kinase receptors with intrinsic catalytic activity (Cosman, 1993).

2.2 The PRL Receptor

PRL receptors are widely distributed throughout the body (Kelly *et al.*, 1993), reflecting the many different functions of this hormone *in vivo*. Within cells, the PRLR is found not only in the plasma membrane, but also in endosomes, and Golgi and lysosomal fractions (Bergeron *et al.*, 1978). Furthermore, PRLR have been characterized or detected in the liver of female rats and rabbits (Haeuptle *et al.*, 1983; Posner *et al.*, 1974), the mammary gland of rabbits (Ymer *et al.*, 1987; Haeuptle *et al.*, 1983), in ovary and adrenal tissue of rabbits, rats, and sheep (Posner *et al.*, 1974) and in milk (Postel-Vinay *et al.*, 1991). The numbers of PRLR vary from tissue to tissue and between species and are, in many cases, developmentally regulated. For example, in rats, PRLR mRNA increases in the liver during pregnancy, and then sharply decreases at the start of lactation (Jahn *et al.*, 1991). In the mammary gland of these same animals, however, PRLR number is low in virgin and pregnant rats, but increases at day 21 of pregnancy and continues to increase through lactation. Furthermore, in the mouse, suckling has been shown to significantly increase levels of the long form of the PRLR (Kim *et al.*, 1997).

Like many hormones PRL can modulate the levels of its own receptor, although the mechanism by which this is accomplished is unknown (Kim, 1997; Dijane, 1979, 1980; Posner *et al.*, 1975). In addition, PRLR receptor levels have been shown to increase in the rat liver in response to GH (Orian *et al.*, 1991).

The PRLR is very important not only to the lactational ability but also to the reproductive development and ability of mice as shown in a recent knockout experiment (Ormandy *et al.*, 1997). Mice heterozygous for the null gene for the PRLR receptor failed to lactate after the first pregnancy due to a highly underdeveloped mammary gland. For subsequent pregnancies, however, does were able to lactate. In contrast, homozygous females for the null mutation were sterile because embryos could not be implanted; therefore, lactation has not been studied in null mutants. These females exhibited a variety of other reproductive abnormalities such as irregular cyclicity, decreased fertilization rates, and the inability to become pseudopregnant. Half of the homozygous males were infertile, while the remaining half had decreased fertility.

2.2.1 Two Main PRL Receptors Identified

To date, two main, naturally occurring, receptors for PRL have been discovered. However, in the rat, one group has detected PRLR of five different molecular weights, each expressed under a different physiological condition (Guillaumot and Cohen, 1994). They identified different forms in the mammary glands of rats during the estrus cycle, in rats injected with E_2 following ovariectomy, and during pregnancy, and lactation. A fifth form of the receptor was present during all physiological conditions, and was a low molecular weight form of about 40 kDa. The significance of these different forms is not known, although this may represent a novel regulatory mechanism for the PRL/PRLR system.

The first of the main forms of the PRLR identified was the short form of the receptor. In the rat, this receptor is encoded for by transcript of approximately 2 kb. The 2 kb form was first identified and cloned in the rat by Boutin *et al.* (1988) and is the predominant type found in the rat mammary gland (Kelly *et al.*, 1991; 1993). Boutin *et al.* (1988) found an mRNA transcript with an open reading frame coding for a protein of 310 aa. The sequence AACATGC is found surrounding the initiation codon, but does not match precisely the Kozak sequence (Kozak, 1986). There is a potential polyadenylation site at position 1414. Theoretically, this transcript yields a 291 aa protein with a M_r of about 33,000.

The second normally occurring form of the receptor is a longer form, varying in length among species. In the rat the long form is 591 aa long with the difference in length being accounted for by a change at aa 262, resulting in a longer cytoplasmic domain (Kelly *et al.*, 1993). It is thought that the two different forms of the PRLR result from differential splicing of the same transcript in different cell types or even the same cell. For example, in the rabbit, there are three major and one minor mRNA transcripts expressed from one PRLR gene (Dusanter-Fourt *et al.*, 1991). These four transcripts mainly differ in the length of their 5' untranslated region (UTR), although some also possess a longer 3' UTR as well (Dusanter-Fourt *et al.*, 1991). The function of the short form of the PRLR is unknown, although it may act as a dominant negative inhibitor of the long form of the receptor (Pierre-Applanat *et al.*, 1997). Short PRLRs can form heterodimers with long PRLRs, thereby inhibiting the activation of second messengers (see 2.24 PRLR Signal Transduction). The short form may also be involved with the mitogenic function of PRL. It has been shown to associate with and activate pp60$^{c\text{-}src}$, and to increase c-fos, c-jun, and c-src expression when bound by PRL (Berlanga *et al.*, 1995).

In humans, only the long form of the receptor transcript, and the long form of the receptor (598 aa) have been identified (Lochnan *et al.*, 1995). This receptor has a predicted molecular weight of about 67 kDa (Boutin *et al.*, 1989). The 5' UTR of the human PRLR is more complex than that of other species, having 6 potential start sites, upstream of the putative initiation codon (Lochnan *et al.*, 1995).

A third form of the receptor has been identified in Nb2 cells, a PRL-dependent rat pre-T lymphoma cell line (Ali *et al.*, 1991). The receptor for PRL found in these cells is of intermediate length, lacking 198 aa (residues 323-520) in the cytoplasmic domain of the long form PRLR (Ali *et al.*, 1992; Rui *et al.*, 1992). The extracellular and transmembrane domains of this intermediate PRLR do not significantly differ from either the long or the short forms (Ali *et al.*, 1992). The intermediate form of the receptor is more sensitive to PRL than the long form, and is fully capable of inducing signal transduction (Ali *et al.*, 1992).

2.2.2 Ligand Binding

The ligand binding domain for the PRLR is located in the extracellular region of the receptor. There are a number of structural features of this domain which appear to be important for the regulation of ligand binding. PRL receptors have five cysteine residues (at positions 31, 41, 70, 81, and 203; Boutin *et al.*, 1988), four of which are in the amino terminal half of the extracellular region (Rozakis-Adcock *et al.*, 1991, 1992). As with the other members of the haematopoietic receptor superfamily, these four cysteines form disulfide bridges that are important for ligand binding. In fact, mutation of any of the first four cysteine residues eliminates ligand binding completely (Rozakis-Adcock and Kelly, 1991). The importance of the fifth cysteine is not known.

As described for the GHR above, in the PRLR seven β-strands are formed anti-parallel to one another in the ligand binding domain, and these seven strands form two ß-pleated sheets lying atop each other as in a sandwich (Kelly *et al.*, 1993).

Several of the other aa's in the cysteine rich region are also important for efficient ligand binding. W24, L38, Y40, and F64 are conserved for all members of the haematopoietic receptor family (Bazan, 1990b), and a number of others are conserved within the GH/PRL receptor family (Rozakis-Adcock and Kelly, 1992). It is speculated that the side-chains of these residues, which are found in the hydrophobic centre of the ß-sandwich, contribute significantly to the conformational integrity of the receptor (Kelly *et al.*, 1993).

Another significant structural component of the ligand binding domain is the WSXWS box. Substitution of any of aa in this sequence results in large decreases in affinity for the ligand, although structural integrity is maintained (Rozakis-Adcock and Kelly, 1992). It is possible that the WSXWS box forms a "floor" or hinge region of the ligand binding crevice for cytokine receptors as predicted by Bazan (1990a). Kelly *et al.* (1993) suggests that this hinge structure prohibit side-chain substitution. A second possibility for the WSXWS box is that it is on the outside of the binding pocket, making it an easy target for binding by accessory proteins, and forming a high affinity binding complex (Kelly *et al.*, 1993). This type of arrangement has been shown for many of the receptors in the haematopoietin receptor family, such as, IL-2, IL-5, IL-6 and GM-CSF (Kelly *et al.*, 1993).

Ashkenazi *et al.* (1987) determined that both oPRL and hGH recognized the same binding sites on the bovine PRL receptor. However, bGH was not able to recognize this site (Gertler *et al.*, 1984).

2.2.3 Expression of the PRLR gene

In general, all PRLR result from a single gene, even in those species that express multiple forms of PRLR. The different forms of the receptor protein seem to result from differential splicing of the primary RNA transcript of the gene. However, these generalisations are not strictly followed in all species. The two relevant species expression systems, rabbit and bovine, will be considered here.

Rabbit PRLR

In the rabbit, four mRNA transcripts for PRLR could be detected by Northern Blot Analysis (Dusanter-Fourt *et al.*, 1991). Three of these are found in relatively high amounts, and are 2.7, 3.4, and 10.5 kb in length. A fourth, minor transcript, is 6.2 kb long. The differences among these four transcripts lie in the 5' and 3' untranslated regions. There are two different but equally expressed 5' UTR's of at least 330 and 390 nucleotides in length (Dusanter-Fourt *et al.*, 1991). Investigation of the 3' end is incomplete, but at least one and potentially more poly-A addition sites exist (Dusanter-Fourt *et al.*, 1991). Each of these transcripts gives rise to a unique precursor to the long form of the PRL receptor. That is, all of the transcripts contain the complete sequence for the long receptor.

Bovine PRLR

The mRNA for the bovine PRLR is also present as a number of different sized transcripts. A major transcript of about 3.8 kb was found in the maternal corpus luteum, intestine, endometrium, and liver (Scott *et al.*, 1992). A second form was found in the corpus luteum and the endometrium only, and is approximately 4.4 kb long (Scott *et al.*, 1992). A third minor transcript has been identified in the corpus luteum only, and is 2.6 kb in length (Scott *et al.*, 1992). Only very low levels of PRLR mRNA transcripts were detected in bovine mammary tissue as compared with levels found in other tissues (Scott *et al.*, 1992).

The bovine cDNA derived from the study by Scott *et al.* (1992) predicts a signal peptide of 24 aa, and a mature protein of 581 aa. As well, five of the seven aa in the region of the predicted methionine start site match those in the Kozak sequence. The extracellular region has two out of the three sites for N-glycosylation conserved among PRLR of other species (Scott *et al.*, 1992). Furthermore, there is a 25 aa sequence within the transmembrane domain which is 92% homologous with this region in the PRL and GH receptors of other species. The bPRLR gene codes for a slightly smaller protein than the human PRLR, owing to a single base pair difference, resulting in a stop codon 41 aa earlier (Scott *et al.*, 1992).

Various attempts have been made to purify and characterize the bPRLR protein, but there are still some discrepancies as to the size of the major protein detected. Ashkenazi *et al.* (1987) estimated the bPRLR to be between 80-85 kDa in size, with a binding subunit of about 37 kDa. That is, they only detected a long form of the receptor.

Smith *et al.* (1993) assayed membrane bound receptors in Holstein cows using hGH as a ligand. They detected a single protein of molecular weight 33-36 kDa. They also saw low levels of a 66 kDa species, which they assumed to be a dimer of the 33 kDa form. They did not detect the long form of the receptor although they conceded that the long form could have degraded during processing. When investigating transcripts for the PRLR no transcript consistent with a short form of the receptor was revealed. This suggests that the 36 kDa form arises from post-translational modifications of the long receptor.

The study by Smith *et al.* (1993) also determined that the levels of bPRLR receptor were developmentally regulated. They found that there was a uniform low level of PRLR in the mammary gland during pregnancy and lactation but reasons for these low levels were not clear. Levels of receptors were found to increase in non-pregnant cows in early lactation.

2.2.4 PRLR Signal Transduction

Until recently the second messenger system for PRLR was unknown. A factor contributing to this lack of information is that there is no primary structural homology between the PRLR and any receptors for which second messengers had been determined (Kelly *et al.*, 1993). Also, second messengers involved in the signal transduction of many other receptor types are not involved with PRLR signalling. For example. Kornberg and Liberti (1989) showed that cAMP was not involved. A number of groups showed some possible links to G-proteins (Barkley *et al.*, 1988; Too *et al.*, 1990; Kilgour, 1996) although the involvement of this class of proteins did not seem to be significant. The possibility that protein kinase C (PKC) is an important PRLR second messenger was also eliminated by Daniel *et al.* (1996). They showed that specific inhibitors of PKC did not reduce PRL induced CAT activity in cells transfected with a construct of the ß-lactoglobulin promoter/CAT gene. Conversely, Rillema and Rowady (1996) showed that PRL induced a transient 5-fold increase in c-fos mRNA in cultured mouse mammary tissue. They theorized that this increase was generated via the PKC pathway, but further study is needed to confirm or refute this hypothesis. However, none of the above studies conclusively elucidated a single, consistent pathway for signal transduction from the PRLR.

JAK kinase involvement

Rui *et al.* (1992) discovered that PRL stimulation caused the PRLR to stimulate the phosphorylation of several proteins on tyrosine residues (see also Waters *et al.*, 1995). One of these, a protein of 120 kDa, was phosphorylated within 60-90 seconds of PRL addition (Rui *et al.*, 1992; Waters *et al.*, 1995), and could be extracted from fractions with the transmembrane receptor subunits (Rui *et al.*, 1992). Rui *et al.* (1992) hypothesized that this 120 kDa protein might be a novel autophosphorylating tyrosine kinase. This hypothesis was later confirmed by Dusanter-Fourt *et al.* (1994) who also demonstrated that the p120 protein is a member of the Janus family of kinases, JAK2. Daniel *et al.* (1996) showed the involvement of JAK2 by inhibiting PRLR signal transduction to the ß-lactoglobin gene by a JAK2 inhibitor, herbimycin A. The association of the PRLR with JAK2 is constitutive, and seems to occur via the Box 1 region of the PRLR (Pezet *et al.*, 1997; Pierre-Applanat *et al.*, 1997). It has also been shown that JAK1 is phosphorylated to a lesser degree on PRL stimulation of BA/F3 cells (Gao *et al.*, 1996) and CHO cells (Ferrag *et al.*, 1996) transfected with the PRLR gene.

The first step in PRLR signalling is believed to be receptor dimerization (Sakal *et al.*, 1997; Fuh *et al.*, 1993; Djiane *et al.*, 1985). Sakal *et al.* (1997) showed that one PRL molecule bound with two receptor molecules. They suggest that the formation of this dimer be either induced by the hormone, or exist prior to hormone binding. Dimerization is thought to bring into close proximity the proline-rich Box 1 regions of the two receptors, and is associated with the activation of JAK2 (Waters *et al.*, 1995). JAK2 can be bound by any of the three forms of the PRLR, suggesting that its binding site is common to all three receptors (Lebrun *et al.*, 1995b). The Box 1 region, with its proline-rich region, fits this requirement, although this theory has yet to be verified.

The binding of JAK2 is necessary for tyrosine phosphorylation of the kinase, the receptor, and STAT1 (Lebrun *et al.*, 1995b). However, the phosphorylation of these molecules alone is not sufficient for PRL-mediated activation of the ß-casein promoter. The association of the receptor with other signalling molecules in the cytoplasmic domain is probably necessary for specific signal transduction. In addition, Lebrun *et al.* (1995b) found that the short form of the receptor, although able to bind JAK2, did not become tyrosine phosphorylated in the presence of PRL, indicating that the regions common to the long and the Nb2 forms of the receptor are necessary for receptor phosphorylation. They found that the interbox region and the carboxyl-terminal regions were needed for full biological signalling to occur.

Receptor phosphorylation is essential for the transduction of the PRL signal. It has been shown that phosphorylation was transient, peaking one minute after exposure to PRL and barely detectable after 10 min in an *in vitro* experiment (Waters *et al.*, 1995). It is thought that this may be due to the activity of phosphatases, such as PTP1C or PTP2. Waters *et al.* (1995) presented some potential reasons for the short activation time of the PRLR. One possibility is that a quick return to the inactive state returns the system rapidly to a basal state, so that the cell is ready for the next hormone pulse. Alternatively, Waters *et al.*(1995) suggested that rapid dephosphorylation of the receptor could be a method for desensitizing the cell to further hormone signals.

Signal Transducers and Activators of Transcription (STATs)

The events following the phosphorylation of JAK2 kinase in PRLR signal transduction are less well understood, although it is now generally accepted that signalling occurs through signal transducers and activators of transduction (STATs). Six STATs have been identified thus far (Gao *et al.*, 1996); all are activated by phosphorylaton on a tyrosine residue. Once phosphorylated, STATs form homo- or hetero-dimers, travel to the nucleus, and bind with specific sites on the DNA, called prolactin response elements (PREs)(Kirken *et al.*, 1997; Kazansky *et al.*, 1995). While STAT1, STAT3 and STAT5 are all activated in response to PRL, STAT5 seems to be the most significant in the PRLR signal cascade (DaSilva *et al.*, 1996; Lebrun *et al.*, 1995a; David *et al.*, 1994).

STAT5 was originally identified as a mammary gland factor (MGF)(Scmitt-Ney, 1991), but has since been classified as a STAT (Wakao *et al.*, 1994; Gao *et al.*, 1996). It was first isolated from sheep mammary gland tissue, and is known to be involved in the regulation of milk protein gene transduction (Jolivet *et al.*, 1996; Gouilleux *et al.*, 1994;

Wakao *et al.*, 1994). Wartmann *et al.* (1996) identified two isoforms of STAT5 in HC11 cells (a cell line derived from COMMA-ID, a mouse mammary epithelial cell line): STAT5a and STAT5b. These two proteins have 96% structural similarity. Kazansky *et al.* (1995) discovered two isoforms of STAT5a, designated STAT5a1 and STAT5a2, which are alternatively spliced forms of STAT5a. These two forms of STAT5a may have different functions, since they were expressed differentially depending on the reproductive stage of the animal (Kazansky *et al.*, 1995). Kazansky *et al.*(1995) suggested that this may represent another form of regulation of PRL function at the signal transduction level.

For STAT5, phosphorylation occurred on Tyr_{694} (Gouilleux *et al.*, 1994). Phosphorylation on this specific residue was essential for STAT5 activation, nuclear translocation, and DNA binding (Gouilleux *et al.*, 1994). In Nb2 cells phosphorylation of STAT5 was detected within one min of PRL stimulation and was sustained for 60 min (Kirken *et al.*, 1997). Wartmann *et al.* (1996) also showed that the two STAT5 isoforms (a and b) were constitutively phosphorylated on a serine residue. This was later confirmed by Kirken *et al.* (1997). Following treatment of the cells with a lactogen, both STAT5a and STAT5b were also tyrosine phosphorylated.

Wakao *et al.* (1994) showed that the highest levels of STAT5 mRNA in sheep were found in the mammary gland, although it was also detected in the ovary, thymus, spleen, kidney, lung, muscle and adrenal gland. Kazansky *et al.* (1995) showed that levels of STAT5a mRNA were detectable in virgin and early pregnant rats, and were highest during late pregnancy. At the onset of lactation mRNA levels dropped precipitously and stayed low throughout lactation. They suggested a "hit-and-run" response in which STAT5a displaced negative regulatory factors from milk protein promoter areas at the start of lactation, allowing other positive factors to access the DNA throughout lactation.

In contrast, Liu *et al.* (1997) showed that although levels of these two proteins were similar in virgin, pregnant, or lactating mice, there were differences in phosphorylation levels. They found that tyrosine phosphorylation was low in virgins but increased sharply during late pregnancy, followed by a rapid decrease during involution. As well, they showed that during lactation only STATs 5a and 5b bound DNA in the mammary gland.

Interestingly, although the two STATs (a and b) are very similar they have slightly different roles. STAT5a knockout mice developed normally, but showed decreased mammary lobuloalveolar outgrowth during pregnancy (Liu *et al.*, 1997). In addition, females were unable to lactate due to a lack of terminal differentiation. STAT5b did not compensate for the lack of STAT5a ability, suggesting a slightly different role for this isoform.

In the ß-casein gene promoter there is a STAT5 binding site located at base pairs -99 to -89 which is essential to induce casein genes: $^{5'}$TTCTTGGAA (Gao *et al.*, 1996). This type of site is often referred to as a PRL response element (PRE). This sequence is very important for activation of a gene by STAT5; similar sequences have been identified in other milk protein gene promoters, such as that for the ß-lactoglobin gene (Demmer *et al.*, 1995). For this gene, it was found that response to PRL decreased as the length of the promoter was reduced, suggesting the presence of multiple PRE's. As well, when the most proximal of these consensus sites was removed, all PRL response was abolished (Demmer *et al.*, 1995).

The presence of the STAT5 binding site conferred PRL responsiveness when placed 5' to a gene that normally does not respond to PRL (Gao *et al.*, 1996). As well, Gao *et al.* (1996) showed that this PRE acted in both directions and that increasing the number of copies of the PRE in the region 5' to a promoter resulted in increased transcription in response to PRL stimulation.

Regulatory binding sites for STAT5 have been identified farther upstream of the promoters for bovine ß-casein (Schmidhauser *et al.*, 1992) and the rabbit α_{s1}-casein (Pierre *et al.*, 1992) genes. Pierre *et al.* (1994) found four sites within the distal regulatory region -3442/-3285 in the rabbit αs1-casein gene. Jolivet *et al.* (1996) investigated one of these sites, F4, more closely and identified it as a genuine STAT5 binding site using CHO k1 cells.

An interesting interaction has been observed between glucocorticoids and PRL action on the PRLR. Stöcklin *et al.* (1996) cotransfected COS cells, which have no endogenous PRLR or STAT5 expression, with the glucocorticoid receptor (GR) gene, the PRLR gene, and the STAT5 gene, and a ß-casein promotor 5' to the luciferase gene. They found that PRL alone produced a 10 fold increase in luciferase activity, whereas glucocorticoid alone had no effect. However, when both PRL and glucocorticoid were added simultaneously, a 40-fold increase was observed. Their study showed that the synergism observed resulted from direct protein-protein interaction between the GR and STAT5, and that this association was independent of DNA binding. They also showed that STAT5 phosphorylation at Tyr_{694} was necessary for the interaction, and that genes with glucocorticoid response elements (GRE's) were down regulated in response to concurrent treatment of PRL and glucocorticoid.

Other Signal Transduction Machinery involvement

Although STAT5 seems to be the major method for signal transduction from the PRLR, many studies have also demonstrated the involvement of other protein kinases, such as Raf and Ras, in PRL signalling. One study examined the possibility that PRL mediated mitogenesis was achieved through the increase of multiple protein kinases in the nucleus (Ganguli *et al.*, 1996). They found that PKC, casein kinase, and protein tyrosine kinase activities in the nuclear fraction were transiently increased in response to PRL in Nb2 cells.

Clevenger *et al.* (1994) showed that all forms of the PRLR were associated with the Raf-1 kinase. In addition, they found that PRL stimulation of Nb2 cells resulted in the dose dependent serine/threonine phosphorylation of both the p72 and the p74 forms of Raf-1. Activation was transient and maximal at 10 min following PRL administration, while activity was maximal at 20 min after PRL addition (as measured by histone H1 phosphorylation).

Raf-1 is the first kinase in a cascade of serine/threonine kinases originating from the phosphorylation of Ras or PKC. Ras in its inactive form is bound to GDP, and in its active form is bound to GTP. Ras does not directly interact with membrane receptors, but is associated via adaptor proteins such as p120 Ras-GAP, VAV or SOS. Erwin *et al.* (1995) treated Nb2-C11 and Nb2-SP cells with PRL for 15 min, and noted a significant increase in the percentage of GTP-bound Ras. This seems to have been due to tyrosine phosphorylation of SHC (a Src homology region-2 domain adapter protein which recruits Grb2 and SOS). These researchers postulated that phosphorylation is mediated by JAK2 activation, but it is not known whether SHC interacts directly or indirectly with JAK2. JAK2 is known to possess consensus sequences for SHC-SH2 domains (Duhe *et al.*, 1995).

3. Prolactin Regulation of Casein Secretion

3.1 Caseins

Milk is composed of many different elements including fat, carbohydrates, and proteins. Proteins make up about 3.3% of milk in the bovine. The main proteins in milk are casein and whey proteins, including ß-lactoglobulin, α-lactalbumin, lactotransferrin, serum albumin, and immunoglobins (Eigel *et al.*, 1984). In bovine milk, caseins comprise approximately 80% of the total protein content (Wong *et al.*, 1996). There are a number of different caseins found in milk: α_{s1}-casein, α_{s2}-casein, ß-casein, and κ-casein. All casein genes are found in a cluster within less than 200 kb on chromosome 6 and code for proteins that are amphiphilic and form micelles in solution (Wong *et al.*, 1996). Caseins are phosphoproteins which all have phosphorylation sites at a Ser-X-A site, where A is a glutamine or phosphorylated serine, and X is any amino acid. These Ser-X-A sites are clustered in the N-terminal region of caseins (Wong *et al.*, 1996). α_s-Caseins precipitate at low pH (4-5) due to their phosphate content (Wong *et al.*, 1996).

3.2 Regulation of Casein Secretion

Choi *et al.* (1988), using a system of cultured bovine mammary alveoli, found that the proportions of casein mRNA's--40% α_{s1}-CN, 12% α_{s2}-CN, 37% ß-CN, and 11% k-CN mRNA--corresponded closely to the proportions of caseins found in milk. They concluded that synthesis of these proteins was directly dependent on the amount of mRNA present and that there was no competition among casein mRNA's for translational machinery.

Casein mRNA expression is regulated in a complex way and involves many hormones, the differentiation state of the cell and the physiological state of the mammary gland (Shamay *et al.*, 1989). For instance, Shamay *et al.* (1987) showed that casein sythesis could be significantly inhibited by progesterone at pharmacologic doses in lactating tissue. Glucocorticoids have been shown to enhance the induction of casein genes by PRL in a number of different studies (Choi *et al.*, 1988; Doppler *et al.*, 1989; 1990; Schmitt-Ney *et al.*, 1991).

A variety of different methods have been employed to study these mechanisms of regulation including *in vitro* methods, such as mammary gland explants, mammary alveoli cultures, and cell lines such as the HC11 and Nb2 cell lines. A few studies have also included *in vivo* experiments.

3.2.1 ß-Casein Gene Regulation

Many studies involving regulation of milk protein genes by PRL have focused specifically on the regulation of ß-casein expression. Doppler *et al.* (1989), using HC11 cells transfected with a ß-casein promoter/CAT gene construct, found that expression of the CAT gene was significantly enhanced by the addition of PRL. Insulin had no effect on CAT production (see also Choi *et al.*, 1988), PRL or glucocorticoid alone caused a four-fold induction, and PRL combined with glucocorticoid increased CAT production by 37 times. Choi *et al.* (1988) found a less dramatic induction of ß-casein using mammary alveoli (4.7-fold increase with PRL and hydrocortisol treatment). They suggested that the glucocorticoid may increase PRLR binding capacity, either by changing the affinity of these receptors for PRL or by increasing the number of receptors (Akers, 1985). Doppler *et al.* (1990) found that dexamethasone (DEX) increased the sensitivity of HC11 cells to PRL, and that this increase in sensitivity was reversible. Dexamethasone was found to act over a long time period (peak effect at 5 days), whereas PRL had a rapid effect on endogenous ß-casein gene induction (hours to maximum response). They also found that cells that were not pre-treated with DEX showed very weak induction of ß-casein by PRL (see also Teyssot and Houdebine, 1981). They suggested that the action of DEX may be to increase transcriptional activation and be probably an indirect effect since there is no glucocorticoid responsive element in the ß-casein promoter region.

Doppler *et al.* (1989) found that the induction of the ß-casein promoter in HC11 cells was dependent on cell density and that the greatest effect was attained three to four days after the cells had reached confluence. This phenomenon has also been seen in other studies (Doppler *et al.*, 1990; Schmitt-Ney *et al.*, 1991), but the reason for this dependence on confluence is not known.

There are multiple sequence elements necessary for response to hormones within the ß-casein promoter (Doppler *et al.*, 1989). Using a cell line that is dependent on both extracellular matrix and PRL for differentiation (COMMA-ID 9), Schmidhauser *et al.* (1990) demonstrated that expression of ß-casein depended on the length of its promoter. More specifically, Doppler *et al.* (1989) found that, in HC11 cells, the presence of the region from -180 to -265 was needed for modest response to PRL, whereas the region from -265 to -285 conferred sharp enhancement of ß-casein induction. They also determined that the -300 to -2300 region was necessary for DEX to have an effect. Within this region there is the consensus sequence TGTTCT at minus 510 in the rat ß-casein gene; deleting this region resulted in loss of DEX inducibility.

Schmitt-Ney *et al.* (1991) found that mammary gland cells expressed a tissue specific nuclear factor which they named the Mammary Gland Factor (MGF). This factor was later found to be a STAT protein. They found that this factor bound to highly conserved regions within the ß-casein promoter. This sequence, TTCTTGGAATTAA is conserved among species for both the α_{S1}- and ß-casein genes. Other nuclear binding factors--A, B, C, and D--were also involved in the regulation of the ß-casein gene in this study. Schmitt-Ney *et al.* (1991) found that factors A and B formed a complex that bound specific regions within the ß-casein promoter acting to suppress transcription. Mutation of these binding regions increased ß-casein gene expression. After hormone induction, complex C replaced the A/B complex. They concluded that part of the mechanism of control of this gene was lifting transcriptional depression by these nuclear factors.

3.2.2 α_{S1}-Casein Gene Regulation

In contrast to what is known about the regulation of the ß-casein gene, relatively little is known about the control of the α_{S1}-casein genes. Choi *et al.* (1988) found no induction of α_{S1}-CN mRNA in mammary alveoli treated with PRL alone or with PRL and hydrocortisol. However, the presence of consensus sequences in the α_{S1}–casein gene promoter for the binding of MGF (STAT5) has been confirmed in other studies (Jolivet *et al.*, 1996; Pierre *et al.*, 1994). As well, significant induction of the α-casein promoter has been observed in a number of *in vitro* studies. Pierre *et al.* (1992) found that α_{S1}-casein was undetectable when primary rabbit MG cells were cultured with insulin and cortisol, but PRL produced a large increase in the mRNA for this protein. They found that the presence of a distal 5' element (-3768/-3155) within the α_{S1}-casein promoter was important for the strong induction of this gene by PRL. In a subsequent study, Pierre *et al.* (1994) found that this distal 655 bp element (-3772/-3118) interacted with a proximal promoter region (-391/-51) to produce a high level of PRL sensitivity. Within this proximal promoter region is an MGF-like binding site that is very important to the interaction between the distal and proximal regions: a double mutation in this region produced a significant decrease in response to PRL (Pierre *et al.* 1992).

In addition to the presence of an MGF binding site within the proximal region of the α_{s1}-casein promoter, four binding sites for MG nuclear factors have been identified within the distal enhancer region designated F1, F2, F3, and F4 (Pierre *et al.*, 1994). Jolivet *et al.* (1996) examined these distal binding sites and found that F4 (-3333/-3307) was a genuine MGF (STAT5) binding site and was necessary for maximal induction of the promoter.

4. Significance of this Research

Most studies investigating how PRL regulates milk protein production have involved the use of rabbit, rat or human systems. The involvement of PRL in these species is relatively similar in that this hormone is needed for both the initiation and the maintenance of lactation (Flückiger and Wagner, 1968; Canales *et al.*, 1976). However, in ruminants, the role of PRL is less well understood, and its involvement seems to be different from rabbit, rat and human systems. That is, the role of PRL is involved in initiating milk production in ruminants, but its role in maintenance of lactation is unclear (Gertler *et al.*, 1982). For example, when injected exogenously, PRL did not increase the production of milk in cows or goats (Byatt *et al.*, 1997; Plaut *et al.*, 1987), whereas in other species exogenous injections of PRL resulted in augmented milk secretion (Cowie, 1969; Bass *et al.*, 1974). For this reason, studies not involving species other than bovine or caprine cannot be directly applied to cows or goats. One hypothesis for the lack of response of the ruminant mammary gland to exogenous PRL is that the number of receptors on the mammary epithelial cells is insufficient to respond to high levels of PRL.

Until recently, the study of lactation in ruminants has involved the use of *in vivo* techniques, or animal tissue in *in situ* experiments such as mammary gland explants or mammary alveoli. However, the development of a bovine mammary epithelial cell line, the Mac-T cell line (Huynh *et al.*, 1991), has opened the possibility for *in vitro* studies into various aspects of the maintenance of lactation in ruminants. Most, if not all, mammary epithelial cell lines are unable to produce milk proteins, which could be due to a lack of PRL receptors.

In order to investigate the role of the PRL receptor in the regulation of cell proliferation and milk protein synthesis the Mac-T cell system was used. This study aimed to determine if overexpressing PRLR in the Mac-T cell line resulted in an increase in cell proliferation and α_{S1}- and β-casein synthesis in response to lactogenic stimulation. If upregulation of milk protein promoters can be obtained with these cells, *in vitro* production of a wide variety of proteins would be possible. Further, the information obtained with this cell line could be useful for the design of *in vivo* experiments.

Objectives

The objectives of this research were to:

1. establish a stable line of Mac-T cell transfected with the PRLR gene and characterize this cell line;

2. examine the effects of increased expression of the PRLR on cell proliferation:

3. study the effects of increased expression of the PRLR on STAT5 and its phosphorylation levels. cell differentiation and casein synthesis.

Materials and Methods

Hormones and Reagents:

Rabbit PRL was generously supplied by Dr. A.F. Parlow of the Harbour-UCLA Medical Centre (Torrance, CA). Insulin was purchased from Gibco/BRL (Burlington, ON) and hygromycin from Bohringer Mannheim (Laval, QC). Hydrocortisol and ovine PRL were purchased from Sigma-Aldrich Canada, Inc (Missisauga, ON). Unless otherwise noted, all reagents were purchased from Sigma-Aldrich, Canada.

Cell Culture:

Mac-T cells (clone 11A) were grown in Dulbecco's Modified Eagle's Medium (DMEM) plus 10 % Fetal Calf Serum (FCS) (Gibco/BRL), 5 µg/mL insulin, 50 mg/L gentamycin (Gibco/BRL). Fifty µg/L hygromycin were added to the medium for transfectants. Cells were maintained in an incubator at 37°C with 5 % CO_2. Cells were cultured and passed in 150 cm^2 tissue culture (Corning/Costar, Cambridge, CA) flasks unless otherwise stated.

Cell Transfection:

The rbPRL receptor plasmid was generously supplied by Dr. B. Pertridou of the Institut National De la Recherche Agronomique, France. The plasmid contains the SV40 origin of replication, a polyadenylation signal, the β-globin introns, the CMV promoter and enhancer, and the cDNA for the rbPRL (2kb) (Edery *et al.*, 1989).

Cells were transfected using the lipofection technique. Cells were plated at 5.0×10^5 cells per 100 mm dish (Sarstedt, St. Larent, QC). The following day, plates were washed for 30 min in DMEM without serum. A 1:1 DNA:lipid (DODAC/DOPE; Inex Pharmaceutical Corporation, Vancouver, B.C.) mixture was prepared. Nine µg of rabbit

PRL-R DNA were used per 100 mm dish. To prepare the DNA:lipid mixture, 5.6 nmole lipid/µg DNA was mixed with double distilled water to make 100 µL. Rabbit PRLR DNA and hygromycin resistance plasmid (1/10 of total volume of rabbit PRLR plasmid) DNA were mixed with DMEM to make 500 µL per dish. Lipid and DNA solutions were mixed and vortexed at maximum speed for 10 sec. Then, the solution was incubated at room temperature for 30 min and the volume was increased with DMEM to 10 mL per dish.

Medium was removed from the washed plates and replaced with the above mixture. Plates were then incubated overnight at 37°C with 5% CO_2. The next day, the DNA mixture was removed, and complete media were added. Selection (hygromycin, 50 µg/L) was applied 24 h later. Clones were isolated using ethanol sterilized cloning rings.

FACS Analysis:

Cells were grown to 70-80% confluence in 25 cm^2 tissue culture flasks (Sarstedt), washed with cold DPBS (Gibco/BRL), and trypsinized with 2 mL of 0.05% trypsin-EDTA (Gibco/BRL) to lift them from the flask. Trypsin was then neutralized with DMEM containing 10% FCS. The cells were counted and 200,000 cells were taken. These 200,000 cells were centrifuged at 3000 x g for 3 min, then resuspended in 200 µL FACS MIX (DPBS with 2% FCS). The cells were incubated for 30 min at 4°C with periodic agitation with 2 µg of the anti-PRL receptor monoclonal antibody, U5 (Affinity Biochemicals, Golden, CO). The reaction was stopped by the addition of 1-2 mL of cold FACS MIX. The reaction mixture was centrifuged as above and the liquid aspirated. Cells were resuspended in 200 µL of cold FACS MIX and 5 µL (1 mg/mL) of FITC conjugated donkey anti-mouse secary antibody (Sigma Immuno Chemicals). Tubes were protected from light from this point onward. Following incubation with the secondary antibody for 30 min, the reaction was stopped by the addition of 1-2 mL cold FACS MIX. Cells were then centrifuged and the liquid aspirated. Cells were resuspended in 750 µL of FACS MIX containing 1% formaldehyde and maintained at 4°C until they were analyzed by flow cytometry. Flow cytometry was performed using a FACScan machine from Becton-Dickinson (Franklin Lakes, New Jersey).

FACS Sort:

Cells were sorted according to their level of fluorescence using a flow cytometry sorting machine (FACS Vantage machine, argon ion laser, wavelength 488 nm; Becton-Dickinson). A minimum of 3,000,000 cells was prepared according to the above protocol, however, in the final step, formaldehyde was omitted from the suspension mixture. Cells were collected in DMEM with 10% FCS and immediately introduced into 25 cm^2 tissue culture flasks. Cells were sorted into three groups: high (H), medium (M), and low (L) levels of fluorescence.

Radioiodination:

Radioiodination was performed using the Chloromine-T method (Lesniak *et al.*, 1973). Five µg of hormone (rbPRL or oPRL) were iodinated in a total volume of 50 µL using 1 mCi $Na^{125}I$ (Amersham, Oakville, ON) and 2 µg of Chloromine-T for 2 min and then separated on a Sephadex G-100 column.

Binding Assays/Scatchard Analysis:

Cell extracts were prepared by scraping 5-10 100 mm dishes (80% confluent) in TM buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) at 4°C. Membrane fractions were prepared by 3 consecutive 5 min freeze/thaw cycles. Extracts were then centrifuged at

9500 x g for 2 min. Supernatants were discarded and pellets resuspended in 500 µL of fresh TM buffer. Protein concentrations were determined using the method of Bradford (Bio-Rad. Mississauga. ON).

Binding assay (BA) buffer was 25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.1% BSA, and 0.02% NaN_3. One hundred µg of each sample was incubated with 50000 cpm ^{125}I-PRL (rabbit or ovine) with or without 200 ng cold hormone in a total volume of 500 µL. Reaction mixtures were agitated at room temperature overnight. After incubation, 500 µL of cold BA buffer were added to stop the reaction, and samples were centrifuged at 3000 x g for 10 min at room temperature. Supernatants were discarded and pellets washed twice with BA buffer. Radioactivity of each sample was counted using a 1282 Compugamma CS Universal Gamma Counter from LKB Wallac (Gaithersburg. Maryland).

For the Scatchard analysis, 100 µg of each sample was incubated with 50.000 cpm ^{125}I-PRL in the presence of 0, 0.5, 1.0. 2.5. 5.0. 10, 50. 100, or 200 µg cold hormone. Data from these experiments were analysed using the program LIGAND (Manson and Rodband, 1980). Each treatment was performed in triplicate.

Growth Curve Analysis:

Mac-T cells transfected with the hygromycin gene (Mac-T_H) and Mac-T's transfected with both the hygromycin and the rbPRLR gene (S15H and SPH) were plated at a density of 2 x 10^4 cells/well in a 24 well plate (Corning/Costar) in 1 mL of complete medium containing only 0.5% FCS (quadruplicate wells). These cells were incubated with or without 5 µg/ml of PRL. Following plating, samples were collected every 24 h for 6 days as follows: ^{3}H-methylthymidine (ICN-Biomedical, Montreal, QC) was diluted in serum-free DMEM to a final concentration of 1.5 µCi/100 µL. At each time point, 100 µL of this solution was added to each well. The plates were then incubated at 37°C for 90 min. Following incubation, medium was aspirated and the plate was air-dried. One mL of fixing solution (3:1 = methanol:acetic acid) was added to each well and incubated for 5 min at room temperature. Fixing solution was then removed and cells washed four times with DPBS. Then, plates were air-dried for 10 min. Five hundred µL of 0.2 N NaOH were added to each well, and plates incubated at room temperature overnight. The next day, 400 µL of liquid from each well was mixed with 4 mL of scintillation cocktail in a scintillation vial (Fisher). The amount of ^{3}H-methylthymidine incorporated was measured (counts per min) using a 1209 Rackbeta Liquid Scintillation Counter from LKB Wallac.

Western Analysis for PRLR

Samples of membrane fraction preparations (100 µg protein) in the sample buffer (5 mM Tris-HCl, pH 6.8, 10 mM β-mercaptoethanol, 0.4% sodium dodecyl sulfate (SDS), bromophenol blue, and 1% glycerol) were loaded into wells of a reducing polyacrylamide gel (4% stacking gel, 8% separating gel). Gels were then electrophoresed at 100 V for approximately 2 h. Size-separated proteins were transferred to nitrocellulose paper (Bio-Rad) at 100 V for 1 h at 4°C or overnight at 20V. The blot was then blocked with antibody incubation buffer (AIB, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.05% Tween-20, 1% BSA, and 0.01% sodium azide) for 2 h at room temperature. This was followed by incubation with the monoclonal antibody, U5 (2 µg/mL) in AIB for 1 h. Blots were then incubated with anti-mouse IgG conjugated with horseradish peroxidase (Promega, Madison, WI) in antibody incubation buffer at a dilution of 1:5000 for 30-45 min at room temperature. Following incubation with antibodies, blots were washed 3-5 times with the wash buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.05% Tween-20), 5-10 min each. Proteins were detected using enhanced chemiluminescence (ECL Kit, Amersham) following manufacturer's instructions.

Immunoprecipitation and Western for STAT5 protein:

To determine whether the increase in PRLR of Mac-T cells affected the expression of STAT5, Mac-T_H, SPH, and S15H cells were grown in 100 mm tissue culture dishes until 70-80% confluent. These cells were then treated for varying lengths of time (ranging from 5 minutes to 120 h) with oPRL (5 µg/ml) in growth medium containing only 0.5% FCS. Similar experiments were performed to investigate phosphorylation of STAT5 in response to PRL.

Cells were scraped from 100 mm dishes in 1 mL of the lysis buffer (10 mM Tris-HCl, pH 7.6, 5 nM EDTA, 50 mM NaCl, sodium - 200 mM sodium orthovanadate, 1 % Triton X-100, 1 mM PMSF, 5 µg/mL aprotinin and pepstatin, and 2 µg/mL leupeptin). Five hundred mL of each sample was incubated with 2 µg anti-STAT5b antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h at 4°C. Antibody-protein complexes were purified by incubation with protein A-sepharose 4B Fast Flow beads overnight at 4°C. Supernatants were removed and beads washed two times with lysis buffer. Beads were then boiled in 40 µl of SDS sample buffer for 5 min and electrophorezed on an 8% reducing polyacrylamide gel (4% stacking gel, 8% separating gel). Blots were transferred to nitrocellulose paper at 80 V for 2 h at 4°C. After transfer, blots were equilibrated in TBS-T (0.2 M Tris-HCl, pH 7.6, 1.37 M NaCl) for 15 min, then blocked in blocking buffer (5% milk in TBS-T) for 2 h at room temperature. Following blocking, blots were incubated with anti-phosphotyrosine antibody 4G10 (0.25 μg/mL, Upstate Biotechnology, Lake Placid, NY) in 2.5% milk in TBS-T (antibody buffer). Blots were then incubated with HRP-conjugated goat anti-mouse IgG (Promega) in antibody buffer (1:20000) for 30-45 min at room temperature. Blots were washed 3-5 times in TBS-T for 5-10 min each. Proteins were detected by enhanced chemiluminescence as above.

Following detection of tyrosine phosphorylated proteins, blots were stripped in stripping buffer (62.5 mM Tris-HCl, 100 mM NaCl, 2% SDS, 100 mM β-mercaptoethanol) at 50°C for 45-60 min. Blots were then re-blocked as above. They were then incubated with anti-STAT5b anti-sera (1:3000) in antibody buffer for 1 h at room temperature followed by goat anti-rabbit IgG (1:10000) for 30-45 min at room temperature. Blots were then washed as above and proteins detected by enhanced chemiluminescence as above.

Differentiation:

Cells were differentiated according to Huynh *et al.* (1991). Briefly, cells were grown to 60% confluency in 15 cm^2 tissue culture flasks (Corning/Costar), in complete medium with or without 1 μg/mL hydrocortisone. The cells were then trypsinized and plated at $2x10^5$ cells per square centimetre on prepared collagen gels in 6-well plates (Corning/Costar) with or without cultured fibroblasts (1×10^6 per well). Duplicates of each sample were plated in 6-well plates without collagen gels (plastic). After 24 h, gels were floated and medium was changed to differentiation medium (complete medium containing 1 μg/mL hydrocortisone and 5 μg/mL oPRL). Medium was then collected every 24 h. Cell extracts were prepared at the end of the experiment by freeze-thaw cycles in TM buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$).

DAS-ELISA for α_{S1}-casein:

ELISA plates (96-wells, Corning/Costar) were coated with 100 µL of anti-α_{S1}-casein (gift from Dr. C.W. Beattie, USDA) diluted 1:10000 in carbonate buffer (0.01M $Na_2CO_3 \bullet 10H_2O$, 0.04 M $NaHCO_3$, 0.5% Thimerosal). Plates were then incubated overnight at 4°C. Following overnight incubation, plates were washed 4 times with PBS-T (0.0025 M KH_2PO_4, 0.04 M K_2PO_4, 0.5% Thimerosal, 0.15 M NaCl, 2% Tween-20). Following washing, plates were blocked with 1% gelatin (w/v) in PBS-T for 30 min at 37°C. After blocking, plates were washed 4 times with PBS-T. Samples and standards were then added to wells and incubated for 1.5 h at 37°C. Plates were again washed 4 times with PBS-T. Then, 100 µL rabbit antiserum (gift from Dr. C.W. Beattie, USDA) diluted at 1:2500 in PBS were added to each well and the plates incubated at 37°C for 1.5 h. Plates were washed 4 times with PBS-T. Finally, 100 µL of alkaline phosphatase conjugated goat anti-rabbit IgG diluted to 1:10000 was added to each well and incubated at 37°C for 1.5 h. After washing the plates 4 times with PBS-T, the amount of secondary antibody fixed was detected by adding 100 µL of 1 mg/mL p-nitrophenyl phosphate disodium salt in 0.1 M diethanolamine buffer (1 M diethanolamine, 0.0001 M zinc acetate, 0.001 M $MgCl_2$, 0.5% Thimerosal pH 9.8). Plates were read at 405 nM using a Labsystems/Multiskan MCC/340 microplate reader (Franklin, Massachusetts).

Statistical Analyses:

For all statistical analyses a P value of < 0.05 is defined as being statistically significant. Statistical analyses were performed by ANOVA using the program SigmaStat (SPSS Inc., Chicago, IL)

Results

Selection and Sorting by FACS Analysis:

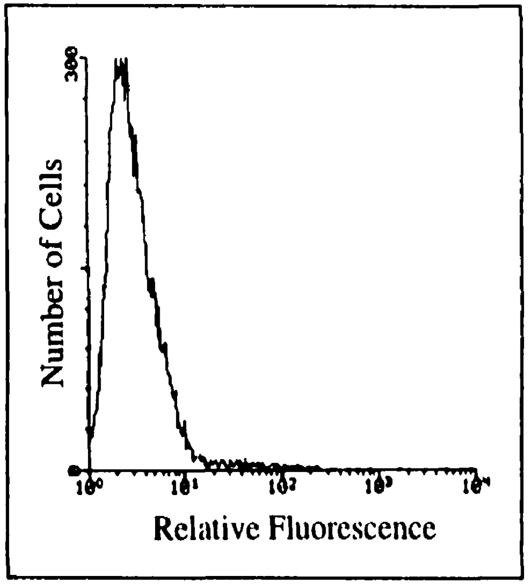
Fifteen clones and a total population (pool) were established following lipofection of Mac-T cells with the rabbit PRLR gene. These clones were analyzed for the expression of the receptor using FACS analysis. One clone (S15) and the pool (SP) showed increased fluorescence relative to the parental cells (Figure 2). The SP and S15 cells were then sorted by FACS into three groups--high (H), medium (M), and low expressors (L)-- based on fluorescence. Only high expressors of the PRLR gene (SPH and S15H) were used for subsequent experiments. Parental cells used in all experiments were transfected only with the hygromycin resistance gene (Mac-T_H).

Binding Assays and Scatchard Analysis:

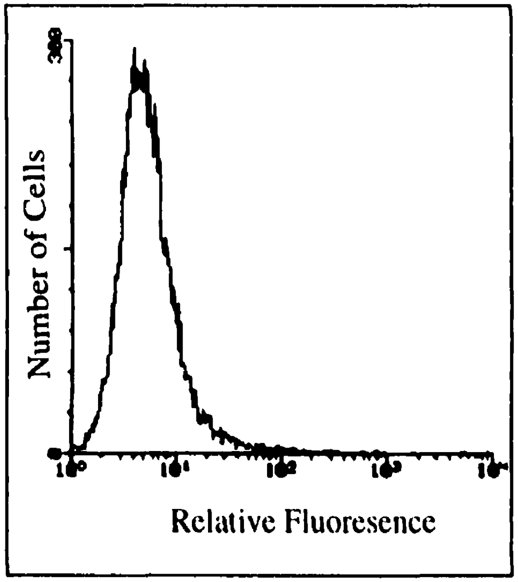
Parental, S15H and SPH cells were analyzed for PRLR binding capacity by binding assays using both rabbit and ovine prolactin as a ligand. Our results showed that rabbit PRL bound with the PRLR about 50% less than ovine PRL (Table 1). There was a significant difference between the binding of rabbit and ovine PRL for the transfectants used in this study, therefore, Scatchard analysis and all subsequent experiments were carried out using ovine PRL.

Specific binding of ^{125}I-PRL for both S15H and SPH was shown to be significantly higher than that for Mac-T_H cells (Figure 3A). As well, Scatchard analysis showed significant levels of binding for S15H cells (Figure 4). No Scatchard analysis could be performed for parental cells due to low binding (data not shown).

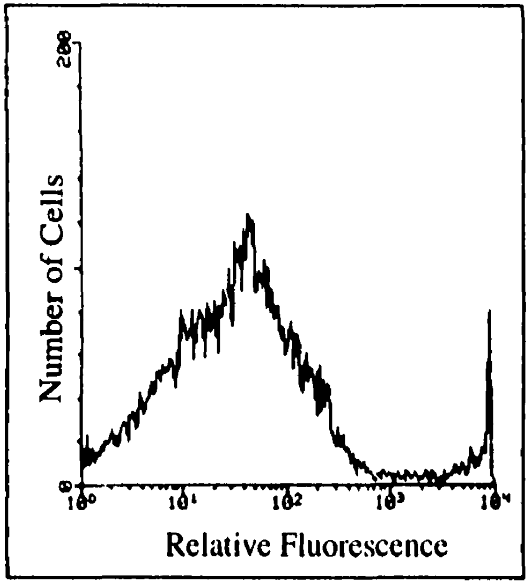
Figure 2: Relative levels of fluorescence of Mac-T_H cells (A), transfectant pool cells (B) and clone S15 (C). Mean fluorescence for parental cells was 5.45 ± 0.015, 8.0 ± 0.3 for SP cells, and 60.1 ± 5.5 for clone S15. There was a statistically significant difference among the three types of cells (ANOVA, $p = 0.011$). The significance was due to the difference between the fluorescence for Mac-T_H and S15 cells (Tukey test; $p < 0.05$) and the difference between SP and S15 cells (Tukey; $p < 0.05$).


300
0
Number of Cells
10^0
10^1
10^2
10^3
10^4
Relative Fluorescence

A.


300
0
Number of Cells
10^0
10^1
10^2
10^3
10^4
Relative Fluoresence

B.


200
0
Number of Cells
10^0
10^1
10^2
10^3
10^4
Relative Fluorescence

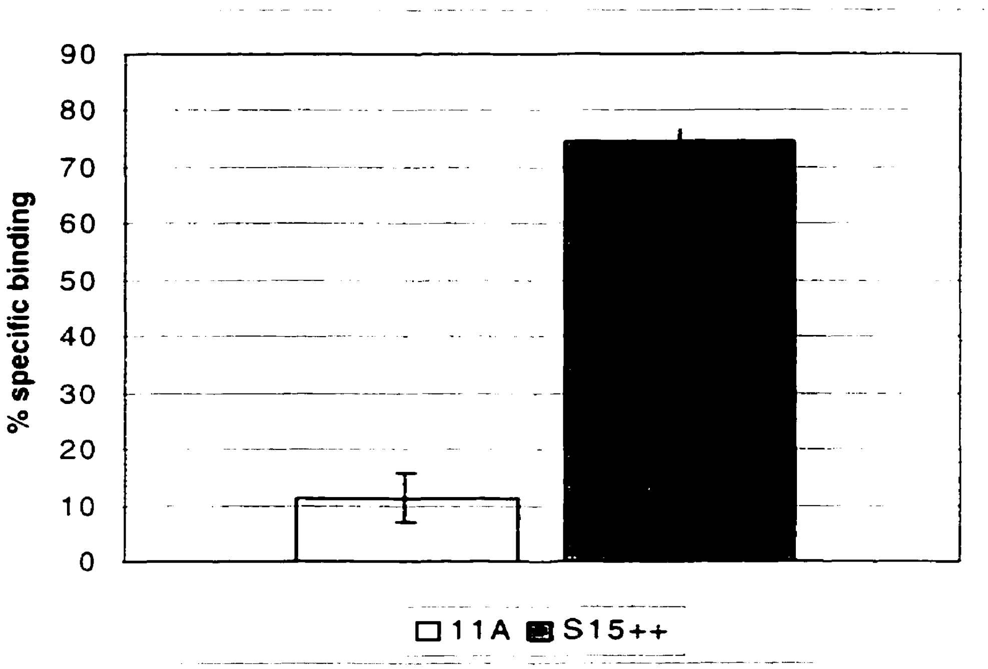
C.

Table 1: Paired one-tail t-test of means comparing specific binding of rabbit and ovine PRL.

	Rabbit PRL	Ovine PRL	P-value
Mac-T_H	5.65 ± 1.21	11.42 ± 4.43	0.25
S15H	31.89 ± 3.34	74.43± 2.16	0.04

Figure 3: Confirmation of the overexpression of the rbPRLR by clone S15H by binding assay and by Western Blot. A. Binding assay. Specific binding for transfectant cells was significantly higher than for parental cells (Student's t-test (one-tailed): $P = 0.033$). B. A Western Blot representating an immunoprecipitation, with monoclonal antibody U5, of the rbPRL from membrane fraction preparations. Nitrocellulose membranes were probed with monoclonal antibody U5, followed by an anti-mouse IgG. An arrow indicates the rabbit prolactin receptor; horizontal lines to the left of the blot show approximate molecular weights. The band at 41 kDa represents the heavy chain of the primary antibody from the immunoprecipitation (U5). Lane 1: Mac-T_H, lane 2: SPH, lane 3: S15H.

A.


90
80
70
60
50
40
30
20
10
0
% specific binding
□ 11A ■ S15++

B.

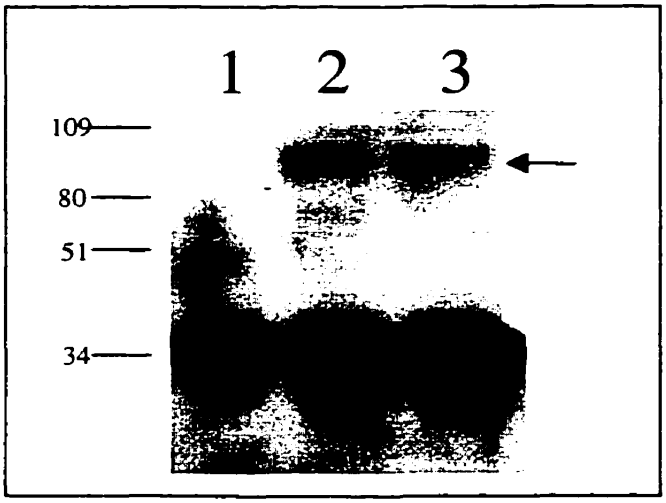
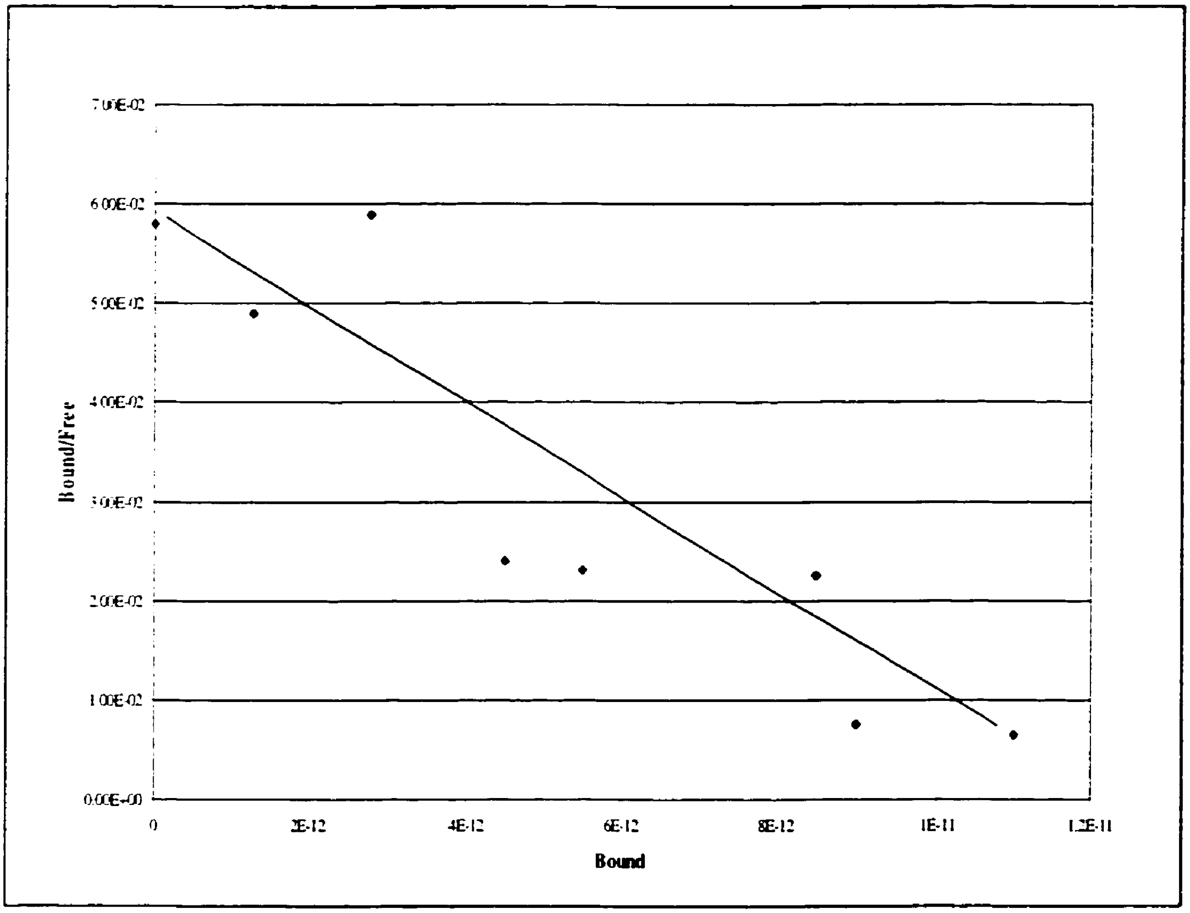

1
2
3
109
80
51
34

Figure 4: Scatchard plot of oPRL binding to membrane fractions of clone S15H cells. Data were from three experiments. $K_d = 3.33 \times 10^9$ nmol/l. $B_{max} = 1.23 \times 10^{-11}$ fmol/mg.


7.00E-02
6.00E-02
5.00E-02
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
Bound/Free
0
2E-12
4E-12
6E-12
8E-12
1E-11
1.2E-11
Bound

Western Blotting:

The presence of the rabbit prolactin receptor in clone S15H and pool SPH was further confirmed by immunoprecipitation of the PRLR followed by Western Blot analysis (Figure 3B). Both immunoprecipitation and Western blotting were performed using the monoclonal antibody. U5 (directed against the rabbit PRLR). No band representing the rabbit PRLR was present for samples obtained from parental cells.

Growth Analysis:

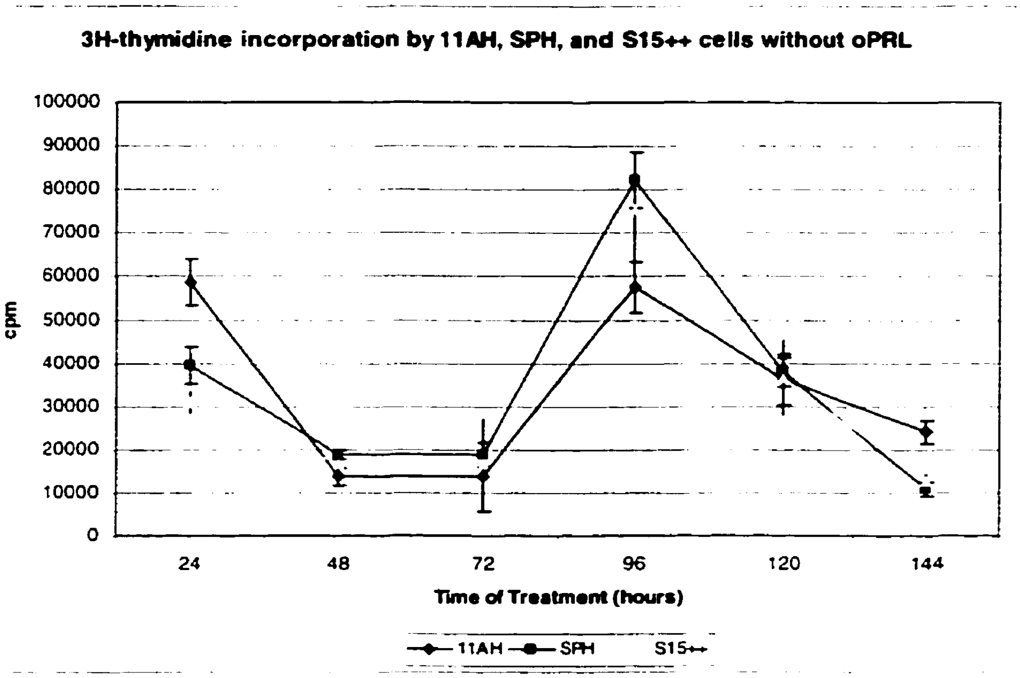
To determine the effect of increased levels of PRLR in Mac-T cells on cell proliferation. ^{3}H-methylthymidine incorporation rates were used. Cells transfected with the PRLR gene were found to incorporate ^{3}H-methylthymidine in a similar manner to parental cells in medium containing 0.5% FCS (Figure 5A). However, in the presence of 5 μg/ml of PRL. both SPH and S15H cells reached the peak rate of ^{3}H-methylthymidine incorporation 24 h earlier than without PRL (Figure 5B). It is interesting to note that the growth of parental cells was inhibited in the presence of PRL.

STAT5 Expression and Activity:

When cells were incubated with or without 5 μg/ml of oPRL for varying lengths of time (5. 15. 30. and 60 minutes), basal levels of STAT5 were not significantly different between parental, pool, and cloned cells (Figure 6A; ANOVA, $P > 0.05$). After prolonged treatment with oPRL, an increase in the relative amount of STAT5 in transfectant and pool cells was noted after 48 h of oPRL treatment (Figure 6B). However. this difference was not significantly higher than the level of STAT5 expressed in parental cells (ANOVA, $P = 0.25$). The relative levels of STAT5 amongst cell types did not change after further treatment with PRL (at 72, 96, and 120 h).

Figure 5: Growth curves for Mac-T_H, SPH, and S15H cells. A: Pattern of ^{3}H-methylthymidine incorporation observed for cells without oPRL in culture medium containing 0.5% FCS. B: Incorporation of ^{3}H-methylthymidine by cells treated with 5 µg/ml of oPRL. Vertical lines indicate standard errors.

A.


3H-thymidine incorporation by 11AH, SPH, and S15++ cells without oPRL
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
cpm
24
48
72
96
120
144
Time of Treatment (hours)
11AH
SPH
S15++

B.

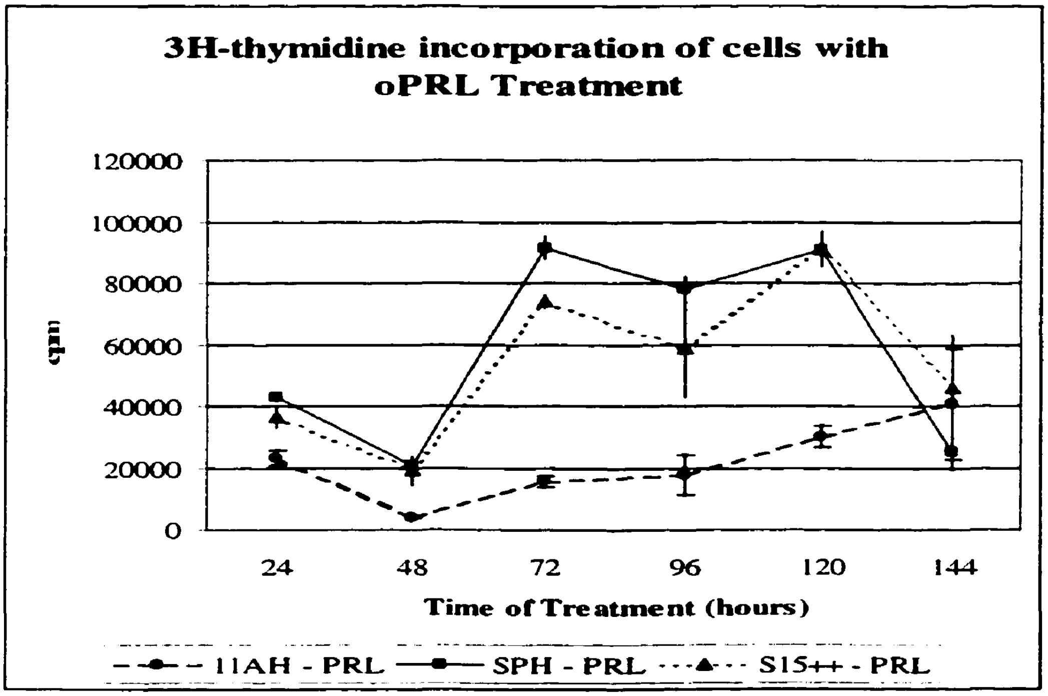
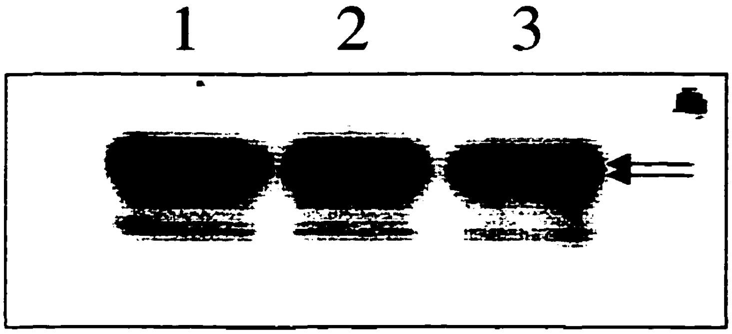

3H-thymidine incorporation of cells with oPRL Treatment
120000
100000
80000
60000
40000
20000
0
cpm
24
48
72
96
120
144
Time of Treatment (hours)
11AH - PRL
SPH - PRL
S15++ - PRL

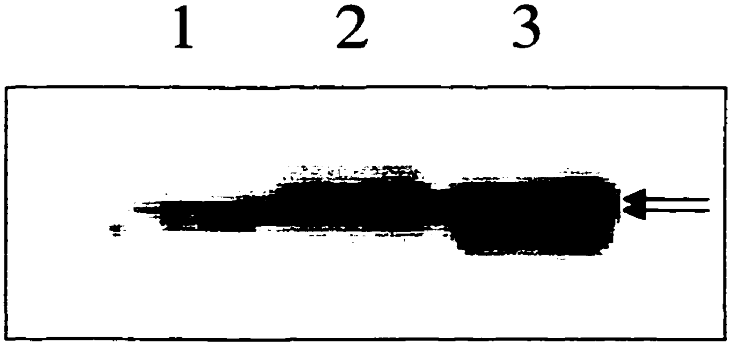
Table 2: Density values for STAT5 expression in Mac-T_H, SPH. and S15H cells.

	Mac-T_H	SPH	S15H
Basal Level	1	0.829	1.016
After 48 h oPRL	1	3.589	7.697

Figure 6: Western blots indicating levels of STAT5 in Mac-T_H (lane 1), SPH (lane 2) and S15H (lane 3) cells. A: Basal levels of STAT5. B: Levels of STAT5 after 48 h of treatment with 5 μg/ml of oPRL. Arrows indicate STAT5 doublet.



A.



B.

The patterns of increase in phosphorylation of STAT5 in response to PRL in Mac-T_H (Table 3 & Figure 7A) and S15H cells (Table 3 & Figure 7B) were similar, with a small increase after 5 min of oPRL stimulation, a peak at 15 min, followed by a decrease in phosphorylation as the time of PRL stimulation increased. However, for SPH cells, a slightly different pattern was demonstrated as phosphorylation levels peaked at 30 min. There was no difference between patterns of phosphorylation for any of the cell types (2-way ANOVA; $P = 0.25$).

Differentiation and production of caseins:

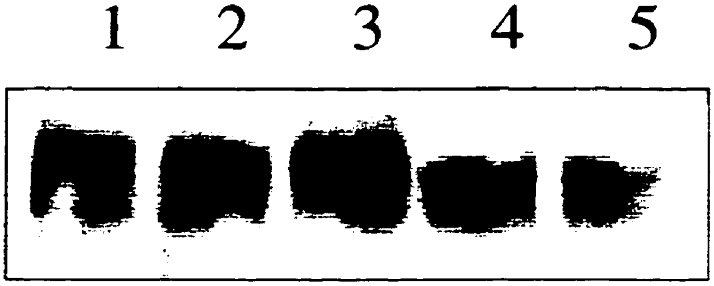
Several methods were attempted to stimulate the differentiation of Mac-T cells and transfectants. These methods included culturing cells prior to plating on collagen with or without hydrocortisone priming, culturing fibroblasts on the collagen gels prior to adding Mac-T cells, and floating or not floating collagen gels with cultured fibroblasts. Based on morphological criteria, none of the three clones were differentiated using any of these methods. Rather, cells remained flat and did not form any three-dimensional structures. Furthermore, no α_{S1}-casein in the medium of any sample well was detectable by ELISA (data not shown). In addition, although β-casein was detected by Western blot in medium collected from the wells, there was no difference between samples (both from cell lysates and from medium) collected from control (non-oPRL treated) and oPRL treated wells, regardless of cell type (Figures 8, and 9). There was no difference between the amount of casein produced by parental cells as compared to the transfectant or to the clone.

Table 3: Density values for STAT5 phosphorylation in Mac-T_H, SPH, and S15H cells.

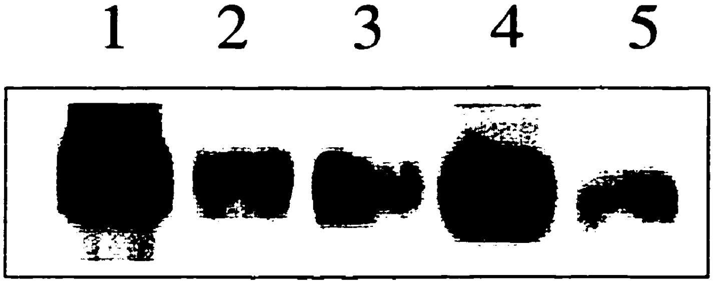
	Mac-T_H	SPH	S15H
Control	1	1	1
5 minutes	1.103	0.342	1.083
15 minutes	1.187	0.355	1.218
30 minutes	0.811	0.835	0.276
60 minutes	0.478	0.207	0.030

Figure 7: STAT5 phosphorylation levels of Mac-T_H (A), SPH (B), and S15H (C) in response to stimulation by 5 μg/ml oPRL. Lane 1: control (no prolactin treatment); lane 2: 5 min; lane 3: 15 min; lane 4: 30 min; lane 5: 60 min.

A.



B.



C.

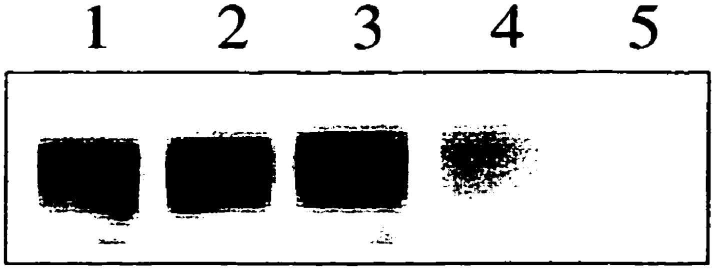
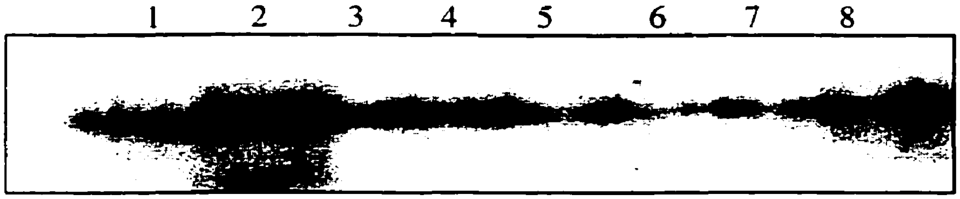


Figure 8: β-Casein expression by Mac-T_H (A) and S15H (B) cells when plated on plastic dishes and stimulated with oPRL. Lane 1: 5 μg β-casein; lane 2: control, 24 h; lane 3: oPRL treated, 24 h; lane 4: control, 48 h; lane 5: oPRL treated, 48 h; lane 6: control, 72 h; lane 7: oPRL treated, 72 h; lane 8: control 96 h; lane 9: oPRL treated, 96 h.

A.



B.

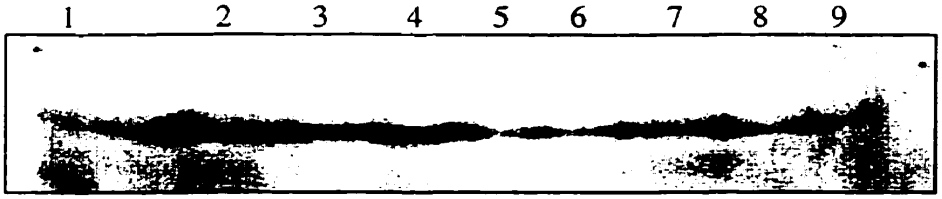
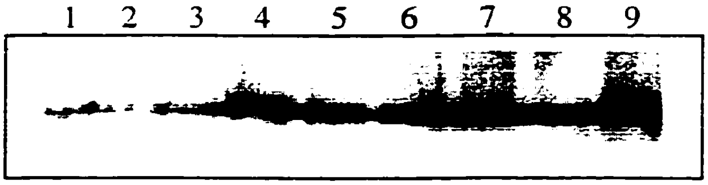
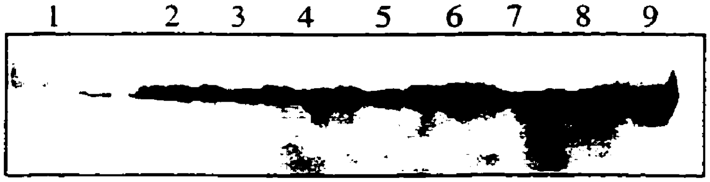


Figure 9: β-Casein expression by Mac-T_H (A), SPH (B) and S15H (C) cells when plated on floating collagen gels and stimulated with oPRL. Lane 1: 5 μg β-casein; lane 2: control, 24 h; lane 3: oPRL treated, 24 h; lane 4: control, 48 h; lane 5: oPRL treated, 48 h; lane 6: control, 72 h; lane 7: oPRL treated, 72 h; lane 8: control 96 h; lane 9: oPRL treated, 96 h.

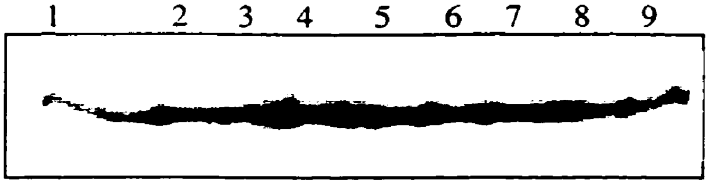
A.



B.



C.



Discussion

Development of a bovine mammary epithelial cell line expressing high levels of PRLR:

In vivo, bovine mammary epithelial cells expressed the short form of the receptor at low levels that varied in affinity for PRL depending upon the reproductive status of the animal (Table 4; Smith *et al.*, 1993). In the same study, no protein corresponding to the long form of the PRLR was detected, although the authors conceded that this could have been a problem of sample preparation. Other studies, however, have detected a long form of the PRLR protein in the bovine (Ashkenazi *et al.*, 1987), and the cDNA sequence predicts a protein of about 557 aa that corresponds to a long form (Scott *et al.*, 1992).

Table 4: Scatchard analysis of the competitive displacement of ^{125}I-labelled GH bound to microsomes prepared from the mammary glands of cows at various stages of the lactation and reproduction cycles. Adapted from Smith *et al.*, 1993.

	K_d (nmol/l)	B_{max} (fmol/mg)	# of Animals
Puberty (NP*)	0.6	22	4
Early Lactation (NP)	7.4	254	3
Middle Lactation (NP)	2.9	32	1
Late Lactation (NP)	7.4	122	4
Late Lactation (P)	1.4	30	4
Non-lactating (P)	1.3	30	4

NP designates non-pregnant animals. P indicates pregnant animals.
K_d = dissociation constant.
B_{max} = binding capacity, fmol ^{125}I-labelled human growth hormone specifically bound/mg membrane protein.

In most species, the long form of the receptor is the only form that transmits PRL's signal to milk related genes in the mammary gland while the role of the short form of the receptor is unknown (Lesueur *et al.*, 1991). This raises the question of the role of

PRL and its receptor in regulating lactation in the bovine. Smith *et al.* (1993) suggested that response to PRL in the cow was regulated by the concentration of receptors on the cell surface rather than by the type of receptor expressed.

Smith *et al.* (1993) also found that microsomes from bovine mammary epithelial cells exhibited low affinity for human growth hormone as compared with ovine, mouse, and rat PRLR. Therefore, a lack of high affinity lactogenic receptors may partially explain why cows are unable to respond to exogenous PRL (Plaut *et al.*, 1997) and why PRL does not seem to play a role in galactopoiesis.

Previously, Mac-T cells, the cell line used for this project, were able to respond to PRL by producing β-caseins under specific conditions (Huynh *et al.*, 1991). Since that time, however, the cells have lost the ability to respond in the same way to stimulation by PRL. One hypothesis that may explain this loss of function is that the cells have lost the ability to express adequate levels of the PRLR. Therefore, the first goal of this project was to establish a stable line of Mac-T cells transfected with the PRLR gene and expressing the PRLR protein. This goal was met by transfecting the Mac-T 11A cell line with the gene for the rabbit PRLR. The rabbit PRLR gene was chosen for several reasons: first, this gene has been previously published and characterized (Edery *et al.*, 1989); second, a monoclonal antibody to the rabbit PRLR was available commercially, whereas antibodies directed against the bovine PRLR are not available; third, the rabbit PRLR receptor codes for the long form of the PRLR, which is known to mediate PRL function with respect to milk protein genes in the rabbit; finally, the predicted amino acid sequence of the bovine PRLR is about 70% homologous with the rabbit PRLR (Scott *et al.*, 1992). One notable difference between the bovine receptor and PRLR of other species is that the bovine receptor gene codes for approximately 47 fewer aa in the cytoplasmic domain (Scott *et al.*, 1992). The significance of this difference is unknown, but it was expected that the rabbit PRLR would be functional in the bovine system.

One of the transfected clones was capable of expressing the rabbit PRLR protein as detected by flow cytometry. Not all amplified clones were expected to express the PRLR since the clones were initially selected based on resistance to hygromycin. As well, other researchers have had difficulty in establishing a cell line stably transfected with this gene (B. Petridou, personal communication), therefore, it is not surprising that we were only able to develop one positive clone.

Sorting the clone and the pool cells enabled us to establish clone and pool cells that expressed relatively high levels of the PRLR protein. The expression of the PRLR protein was confirmed by Scatchard analysis, Western blot, and binding assay. Both rabbit and ovine prolactin were used as the ligand for initial binding assays. These two types of prolactin were used to determine which would be more effective for use in further experiments. A previous study showed that rabbit prolactin rapidly dissociates from the rabbit PRLR in comparison with PRL from other species (Petridou *et al.* 1997). That is, rabbit PRL has a high coefficient of dissociation for its own receptor. This resulted in lower specific binding for rabbit PRL. Our experiments confirmed that our tranfectants specifically expressed the rabbit PRLR since rabbit PRL was found to bind significantly less receptors than ovine PRL.

The above results confirmed the establishment of a cell line stably transfected with the PRLR gene that is capable of expressing high levels of the rabbit PRLR protein on the cell surface. These cells were used to test whether an increase in levels of the long form of the PRLR altered the ability of bovine mammary epithelial cells to respond to PRL.

Effects of PRLR on growth:

The second objective of this study was to determine the effects of the presence of increased PRLR on the growth pattern of transfected cells. *In vivo*, PRL is thought to have two major functions in the mammary gland. One of these functions is its role as a mitogen. The necessity of PRL for normal mammary growth (ductal and lobuloalveolar growth) has been demonstrated in a number of species including mice, rats, and rabbits (Ormandy *et al.*, 1993). In the bovine, the role of PRL in the growth of the mammary is not known although it is believed to be essential for normal development. The results of our study suggested that bovine mammary epithelial cells in culture were responsive to PRL. However, their response to oPRL was different from our expectations. Parental cell growth was inhibited by the presence of PRL, suggesting that PRL may not have a mitogenic effect in this bovine cell line, but rather an inhibitory effect on growth. These cells likely express low levels of the short form of the PRLR as reported by Smith *et al.* (1993) for bovine mammary glands. It is possible that the role of this form of the receptor in the bovine is the inhibitory regulation of cell growth. One study showed that the short form of the PRLR could associate with and activate proteins which are known to be involved in the regulation of mitogenic events, such as pp60$^{c\text{-}src}$ (Berlanga *et al.*, 1995). It was also found that levels of c-fos, c-jun, and c-src were increased when the short form PRLR bound PRL (Berlanga *et al.*, 1995). Furthermore, Ganguli *et al.* (1996) showed increased activities in PKC, casein kinase, and protein tyrosine kinases in response to PRL stimulation of Nb2 cells. It is possible that the normal mitogenic activity of PRL is mediated via short form of the PRLR, and its function is to inhibit growth rather than promote mitogenesis in cultured mammary epithelial cells.

In contrast to the inhibition of growth rate by PRL exhibited by parental cells, transfectant cells (both the clone and the pool) responded positively to PRL treatment. This suggests that the long form of the PRLR is able to confer positive growth response to PRL in cultured bovine mammary epithelial cells.

Another study investigating the effect of various hormones on the growth of Mac-T cells found that PRL had no mitogenic effect on these cells (Woodward *et al.*, 1994). There are several possibilities for the apparent discrepancy between our study and Woodward *et al.*'s study: first, in Woodward *et al.*'s (1994) study, ^{3}H-methylthymidine incorporation was measured only once after an 18 h stimulation period rather than sampling every 24 h over a 6 day period. Second, the cells used in the current study had been transfected with the hygromycin resistance gene, and cells were subsequently cultured in medium containing hygromycin (a eukaryotic protein synthesis inhibitor); this substantially slowed the rate of growth of the Mac-T_H cells. Due to the fast rate of growth of normal Mac-T cells, a difference due to the presence of PRL in culture medium may not have been detectable, whereas an effect was seen with our slower growing cells.

Our study only tested growth of Mac-T_H cells and transfectants at one concentration of PRL (5 μg/ml), which is far above physiological levels (Table 5). This concentration was chosen because it has been used in previous publications to stimulate differentiation in these cells (Huynh *et al.*, 1991). It would be of interest to determine whether similar effects on growth in these cells are seen at physiologic concentrations, as well as whether there is a dose dependent effect of PRL.

Table 5: Serum PRL levels during lactation in the bovine (adapted from Koprowski *et al.*, 1973)

	Early lactation (4 weeks)	Mid-lactation (16 weeks)	Late lactation (after 16 weeks)	Immediately after milking
PRL (ng/ml)	33 ± 4	68 ± 9	53±6	93 ± 9

Effects of PRL on STAT5 expression:

STAT5 is a key protein in the signal transduction pathway of PRL, particularly for its functions in the mammary gland (differentiation and lactogenesis). The role of STAT5 for PRL's function as a mitogen is not known. However, recent knockout mice experiments showed that without the gene for STAT5a, normal growth and differentiation of the mammary gland were severely compromised (Liu *et al.*, 1997). There was little lobulalveolar growth and no terminal end bud differentiation in these mice. The importance of STAT5 to mammary gland function in other species, such as sheep, was inferred by the presence of higher levels of this protein in the mammary gland than in any other organ (Wakao *et al.*, 1996). As well, several *in vitro* studies demonstrated that STAT5 recognition sites in the genes of several milk proteins were necessary for the activation of these genes (Schmidhauser *et al.*, 1992; Pierre *et al.* 1992, 1994; Schmitt-Ney *et al.*, 1991; Jolivet *et al.*, 1996).

In mice it has been shown that levels of STAT5 were similar during all stages of lactation (Liu *et al.*, 1997), however, in rats, mRNA levels were regulated by the reproductive status (Kazansky *et al.*, 1995). Few studies have been conducted to investigate the regulation of the quantity of STAT5 in the bovine, although Yang *et al.* (1996) detected STAT5 in the mammary glands of lactating, but not in non-lactating cows.

The third objective of this study was to examine how the increase in expression of the PRLR affects STAT5 levels and STAT5 activation. Basal levels of STAT5 were unchanged by the addition of PRL to culture medium. However, transfectants expressed higher, though non-significant, levels of this protein after 48 h of treatment with PRL.

The second aspect of STAT5 involvement investigated in this study was that of its activation by tyrosine phosphorylation. STAT5 is activated by JAK2 phosphorylation on Tyr$_{694}$. Phosphorylation of STAT5 occurred within 5-10 min of PRL stimulation in HC11 cells (Gouilleux *et al.*, 1994). It is followed by the formation of homo- and heterodimers that migrate into the nucleus and bind with the DNA of milk protein promoter regions. A study in mice showed that phosphorylation of STAT5 changed with reproductive status of the animal: low in virgins, increasing until late pregnancy, then decreasing sharply at involution (Liu *et al.*, 1997). In a study using cows a positive correlation between STAT5 levels and the productive capacity of these cows was reported (Yang *et al.*, 1997). It was also shown that phosphorylation of STAT5 was maintained in mammary gland explants by the presence of PRL or GH (Yang *et al.*, 1997).

Another consideration is that *in vivo* STAT5 activity is the most prevalent during lactation. That is, most STAT5 activity occurs after the mammary epithelial cells have differentiated. In the present experiment, however, the cells were not differentiated, but merely plated on plastic dishes. Unfortunately, it was impossible to test whether there is a difference in phosphorylation when the cells were differentiated, since the cells could not be induced to differentiate. However, it would be interesting to examine these cells for STAT5 activity again, once they have been differentiated.

Differentiation and production of caseins:

There are several indicators that can be used to confirm whether cells have actually differentiated. For many cell types, the easiest and fastest way to ascertain whether differentiation has occurred is to examine morphology. In the case of Mac-T cells, a characteristic morphology was expected. As described by Huynh *et al.* (1991), Mac-T cells form blisters or secretory dome structures that become connected by duct-like structures as time in culture increases. In the present study, no such structures were observed for any of the treatments designed to induce differentiation.

A second method for determining differentiation is to measure proteins that are produced by differentiated cells only. In the case of mammary epithelial cells, the most likely candidates are caseins. In this study we measured both α_{S1}- (DAS-ELISA) and β-casein (Western Blot) levels. No α_{S1}-casein was detected for any of the cell types under any treatment. The sensitivity of this assay ranges from 2.5 ng to >1000 μg. Therefore, even a very low level of α_{S1}-casein would be detected if it were present. Western blots did not detect β-casein secretion in the culture medium, but there was no difference between control and PRL treated groups.

From these two tests for differentiation it can be concluded that the Mac-T cells used in this study were no longer able to differentiate. There may be several reasons for this lack of response to lactogenic stimuli. First, the cells used in this study were of much later passages than those used in the original study by Huynh *et al.* (1991). It may be that years of cell culture have resulted in the selection of cells that survive and grow well in culture, but do not "remember" how to respond to lactogenic hormones. Second, the cells used here have been transfected with two to three genes (large-T antigen, hygromycin resistance, and PRLR). It is possible that the relatively harsh conditions of the transfection, or the incorporation of the genes themselves played a role in prohibiting the response of Mac-T cells to the induction of differentiation. A third factor to be considered is that the cells used in this study are not from the same clone used in the original study (Mac-T3; Huynh *et al.*, 1991). The cells used here, Mac-T 11A, were a sub-clone developed recently and which may never have been able to differentiate.

There are several other factors that could be involved in inhibiting response to lactogenic stimuli not related to the cells themselves. First, the role played by collagen in cell differentiation is not clearly defined. It is not clear whether only one type of collagen is needed, or whether several must be present for the most efficient induction of differentiation. Second, the optimum concentration of PRL necessary for stimulation of differemtiation was not determined in the original study by Huynh *et al.* (1991), therefore, it is possible that 5 µg/ml is either too high or too low to effectively promote differentiation.

What is the possible biological significance of the fact that PRL inhibits the growth of parental cells yet enhances the growth of transfectant cells? One possible answer is that parental cells are somehow mimicking the situation during lactation *in vivo*. Once differentiated, cells stop dividing; a lack of high affinity receptors may act as protection for the cells from the strong stimulus by PRL to divide. The transfectants constitutively expressed the long form of the PRLR, therefore, were unable to resist the signal from PRL to undergo mitosis. These cells are perhaps representative of mammary epithelial cells in the pubertal and pregnant cow, which express high affinity PRLR and are undergoing rapid growth. The existence of long forms of the PRLR in these physiological stages of the bovine needs to be confirmed.

Another interesting speculation can be drawn from the apparent increase of STAT5 in response to increased signal from PRL: in the presence of increased signal from PRL (as conferred by the presence of increased PRLR) cells are able to synthesize signal transduction machinery to meet the demand caused by the increase in signal. Further testing must be done to more fully understand the biological significance and the mechanism at work in this circumstance. For instance, it is possible that a similar phenomenon may be observed *in vivo*. This increase in STAT5 may be related to PRL concentration or to PRLR concentration. There may be a relationship between the productive capacity of a cow and the level of STAT5 she produces.

Summary

1. A line of bovine mammary epithelial cells expressing high levels of the rabbit PRLR was successfully established. This cell line, Mac-T S15H, exhibited significantly higher specific binding of ^{125}I-oPRL than parental cells.
2. PRL was shown to stimulate Mac-T S15H cells to reach the peak of mitosis earlier than without PRL treatment. PRL was also shown to inhibit the growth of parental cells.
3. STAT5 levels were not different between parental cells and transfectants in the absence of PRL. There was a slight, non-statistically significant increase in STAT5 for Mac-T S15H and Mac-T SPH cells after 48 h of treatment with PRL. Phosphorylation was not different among parental, transfectant or pool cells.
4. Cells were not induced to differentiate, but were shown to produce low levels of β-casein regardless of treatment. No α_{S1}-casein was detectable under any treatment.

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