MOLECULAR BIOLOGY OF AN AUTOCRINE INHIBITOR OF MILK SECRETION

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LIST OF FIGURES

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Chapter Six

LIST OF TABLES

Chapter Two

Chapter Three

Chapter Four

Chapter Six

LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BFA	brefeldin A
CsCl	caesium chloride
DMSO	dimethylsulphoxide
DnD	DMSO & DTT
DTT	dithiothreitol
ECM	extracellular matrix
EGF	epidermal growth factor
EGTA	ethyleneglycol tetraacetic acid
EHS	Engelbreth-Holm-Swarm
FAS	fatty acid synthase
FGF	fibroblast growth factor
FIL	feedback inhibitor of lactation
GAS	interferony activation sequence
GH	growth hormone
GIT	guanidium isothiocyanate
h	hour or hours
$HACoCl_3$	hexamine cobalt chloride
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IPTG	isopropyl-β-D-thiogalactopyranoside
IU	international units
JAK	janus tyrosine kinase
MDGI	mammary derived growth inhibitor
MGF	mammary gland factor
M_r	relative molecular weight
NBT	nitroblue tetrazolium
PAI	plasminogen activator inhibitor
OD	optical density
OLB	oligo labelling buffer
RACE	rapid amplification of cDNA ends
RMYQ	relative milk yield quotient
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSC	sodium chloride, sodium citrate solution
STAT	signal transducers and activators of transcription
TAE	tris, acetate, EDTA buffer
TBE	tris, borate, EDTA buffer
TE	tris, EDTA solution
TEMED	N,N,N',N'-tetramethylethlenediamine
TFB	standard transformation buffer
TGF	transforming growth factor

TIMP	tissue inhibitor of metalloproteinase
T_m	calculated melting temperature
TNT	tris, sodium chloride, tween solution
Tris	tris(hydroxymethyl)aminomethane
WAP	whey acidic protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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LIST OF PUBLICATIONS

The results presented in this thesis have been published in part in the following journals.

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Wilde CJ, Addey CVP, Bryson JM, Finch LMB, Knight CH & Peaker M (1997). Autocrine regulation of milk secretion. In *Mammary Development and Cancer* (Fernig DG & Rudland PH, eds.) Portland Press, London, in press.

DECLARATION

The data presented was collected by myself between October 1991 and May 1995 with the following exceptions. Mammary biopsies were performed by Dr CH Knight and Dr JR Brown (chapter 3). Labelling of 28S RNA and β-lactoglobulin cDNA probes and subsequent hybridisations were performed by Dr LMB Finch (chapter 4). The anti-bovine FIL antibody used for immunoscreening was prepared by F Campbell (chapter 5). With these exceptions I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Dr Colin J Wilde and Dr Caroline VP Addey. No part of this thesis has been submitted for consideration for any other degree or award.

Jane M Bryson

ABSTRACT

Research over many years has shown that the rate of milk secretion is regulated by frequency and completeness of milk removal. The effect of milk removal occurs through local mechanisms within each mammary gland, and recent work indicates that local control is through feedback inhibition by a novel milk protein termed FIL, Feedback Inhibitor of Lactation. Evidence from studies in mammary cell culture suggests that FIL controls the rate of milk secretion, mediating the effect of frequency and completeness of milk removal, by inhibition of constitutive secretion, which involves reversible blockade of mammary membrane trafficking. Due to its effects on membrane trafficking, FIL may also regulate mammary differentiation. This may provide a mechanistic explanation for the developmental changes associated with sustained alterations in milking frequency or efficiency. For example, extended frequent milking elicits a significant increase in secretory cell differentiation as measured by mRNA abundance and activities of key enzymes involved in milk synthesis. However, neither the developmental changes at the level of gene expression or the mechanism underpinning these responses has been characterised in detail.

The aim of this project was therefore to investigate whether frequency of milking does indeed control expression of key milk protein genes and to investigate the mechanisms underpinning the putative regulation of gene expression - specifically, to determine if FIL is competent to influence mammary gene expression.

In the first phase of the project, manipulation of milking frequency and concomitant changes in the rate of milk secretion were found to be accompanied in the long term, but not in the short term, by changes in milk protein mRNA abundance. Treatments which did not change milk yield did not affect milk protein gene expression, indicating that changes in milk protein gene expression, like changes in milking frequency are dependent on effective manipulation of milk removal.

To investigate the molecular mechanisms underpinning the increase in milk protein mRNA abundance, demonstrated *in vivo*, goat mammary cells in primary culture were treated with milk fractions and FIL to determine if this protein was indeed competent to modulate milk protein gene expression. These studies demonstrated that long term exposure to FIL decreases milk protein mRNA abundance *in vitro*, lending further credence to the theory that FIL is a regulator of mammary differentiation.

Changes in gene expression in response to FIL, demonstrated *in vitro*, imply that FIL is involved in the developmental response of the gland to frequency of milk removal. Since FIL is itself a mammary gene product, it is also possible that FIL is an autocrine regulator of its own expression. Therefore, the next phase of this project was to clone the gene for FIL, and, if successful investigate the regulation of its gene expression. Several strategies were implemented to clone FIL including screening of goat mammary cDNA libraries with anti-FIL antibody and with synthetic oligonucleotides constructed on the basis of known FIL protein sequence. These strategies were not successful. Whether this was due to library composition, antibody specificity or excessive redundancy in the predicted nucleotide sequence of caprine FIL remains to be determined.

In conclusion, the project has shown that the developmental responses to frequency and completeness of milk removal are associated with changes in expression of key milk protein genes, and experiments in cell culture suggest these changes may be elicited by FIL, as a long term consequence of its effects on mammary membrane trafficking.

If a man does not keep pace with his companions
Perhaps it is because he hears a different drummer
Let him step to the music which he hears
However measured or far away.

Henry David Thoreau

Chapter One

Review of the Literature

REVIEW OF THE LITERATURE

1.1 Introduction

"Lactation is the final phase of the reproductive cycle of mammals. In almost all species the newborn are dependent on maternal milk during the neonatal period; in most the young are dependent for a considerable time. Adequate lactation is therefore essential for reproduction and the survival of the species and, biologically, failure to lactate can be just as much a cause of failure to reproduce as is failure to mate or to ovulate." (WHO Technical Report, 1965).

Lactation is an integral part of the reproductive process and the primary role of the mammary gland is to produce adequate milk to support the young. The cost to the mother may be high - maternal resources are depleted as her reserves are drawn upon to support the suckling young. Thus, parent-offspring conflict may arise because the long-term interests of each are not identical (Trivers, 1974). Ultimately, the level of investment in each lactation is determined by the mother. Her decision to invest (or not) is based on her long-term reproductive strategy - if she can have another litter quickly she may decide to discontinue investment in the first litter, in order to maximise survival of the next. This decision may not be in the interests of the original litter. Conversely, if she has only one or two young, produced after a long gestation, she may decide to continue investment even at the expense of her own well-being (Peaker, 1989).

The overall investment in lactation is controlled by the endocrine system. Not only is the amount of milk produced critical in the survival of the neonate - milk composition must also be tailored to meet the requirements of the growing young. Milk composition differs considerably between species (Jenness & Sloan, 1970), suggesting that during evolution the composition of milk has been modified to meet the specific needs of the young of each particular species.

1.2 Milk and its components

Milk provides all the nutrients required by the neonate. While the composition of milk varies widely between species, the main components are water, the milk proteins, lactose and fats (reviewed by Mepham, 1987; Davis *et al.*, 1983). In addition to its nutritional role, milk may also play an important role in the transfer of bioactive agents from the mother to her young. Such agents may include immunoglobulins (Telemo & Hansen, 1996), hormones, or hormonally active substances such as growth factors (reviewed by Koldovsky, 1996; Koldovsky & Thornburg, 1987; Peaker & Neville, 1991) which may have regulatory or protective effects in either mother or young.

1.2.1 Lactose

Lactose is the predominant carbohydrate in the milk of most species. Synthesis of lactose takes place in the Golgi apparatus and is catalysed by the enzyme lactose synthase. This enzyme is a complex of two proteins - the ubiquitous galactosyltransferase and the specifier protein α-lactalbumin. Lactose is secreted along

with other aqueous milk constituents in secretory vesicles, by exocytosis at the apical cell membrane. This disaccharide is the major osmole in milk and as such maintains milk volume by influencing water influx into the secretory vesicles.

Alpha-lactalbumin is therefore essential for the production of lactose, and thus for lactation. Recent studies have demonstrated that α-lactalbumin deficient mice produce thick viscous milk containing no lactose (Stinnakre *et al.*, 1994; Stacey *et al.*, 1995). Milk volume in these animals is reduced and their young do not survive. This protein is therefore an excellent marker for the differentiated state of the mammary gland. The synthesis of lactose is reviewed by Kuhn *et al.* (1983), Leong *et al.* (1990), Mather & Keenan (1983) and Vonderhaar & Ziska (1989).

An important regulator of lactose synthesis in the lactating gland is the availability of glucose within the Golgi (Faulkner & Peaker, 1987). In the rat, glucose uptake by the mammary cell takes place via a glucose transporter (Madon *et al.*, 1990). The number of transporters is proportional to milk yield, and these transporters are regulated by prolactin and growth hormone (Fawcett *et al.*, 1991; Hudson *et al.*, 1997). The hormonal regulation of mammary glucose uptake by glucose transporter proteins provides a possible mechanism for the control of lactose synthesis and thus milk production. Another possible mechanism for the control of lactose synthesis exists. A whey protein termed FIL (Feedback Inhibitor of Lactation) is competent to decrease lactose synthesis in explant culture bioassay (Wilde *et al.*, 1995). Thus, local factors

which are produced by and act on the mammary secretory cell may also regulate the rate of lactose synthesis.

An increase in the rate of lactose synthesis around parturition is related to the start of copious milk production. Simplistically, the withdrawal of progesterone and increase in prolactin levels triggers lactose synthesis (Kuhn, 1969). Indeed, administration of progesterone in the rabbit retards the appearance of lactose (Denamur & Delouis, 1972). The endocrine control of lactation will be discussed further in section 1.4.

1.2.2 Fats

Milk fat is composed of a complex mix of lipid, predominantly triglycerides. Milk fat provides the major energy source for the neonate. Milk fat content is extremely variable between species ranging form around 1% in the donkey to over 50% in the milk of the gray seal. Fatty acids used for synthesis of milk triglycerides arise from breakdown of blood lipids or by *de novo* synthesis within the epithelial cell itself. *De novo* synthesis of fatty acids takes place in the cytoplasm and is catalysed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (reviewed by Dils, 1983). Lipid synthesis is induced early in pregnancy and reaches a maximum during lactation. Elaboration into triglycerides takes place on the outer surface of the smooth endoplasmic reticulum. As triglycerides are formed they aggregate into small lipid droplets which fuse, forming large fat droplets, prior to secretion. Once the fat droplets reach the apical surface of the cell they bud directly into the milk, enveloped

by a milk lipid globule membrane derived from the apical membrane (reviewed by Keenan *et al.*, 1992; Keon *et al.*, 1993).

Acetyl-CoA carboxylase catalyses the first committed step of fatty acid synthesis. This enzyme exists in both active and inactive forms and its activity depends on the amount of enzyme and its activation status. ACC is regulated allosterically by fatty acyl-CoA and citrate which respectively inhibit and activate the enzyme's activity (reviewed by Munday & Hardie, 1987). Insulin also plays a key role in the regulation of ACC influencing both dephosphorylation, and thus activation of the enzyme (McNeillie & Zammit, 1982), and ACC mRNA abundance (Barber *et al.*, 1992a).

In addition to the role of insulin in ACC enzyme activation, prolactin and growth hormone are also implicated in the regulation of fatty acid synthesis. Prolactin is involved in both the uptake and synthesis of fatty acids by mammary epithelial cells (reviewed by Vonderhaar, 1987a). In sheep mammary explants prolactin acts to increase both ACC enzyme concentration and activity (Barber *et al.*, 1991). Prolactin is also required for the induction of thioesterase II, an enzyme found exclusively in the mammary gland, which is required for synthesis of medium chain fatty acids in this tissue (Carey & Dils, 1973, Knudsen *et al.*, 1976). Thioesterase II activity is closely related to circulating levels of prolactin: enzyme levels increase during gestation, peak during lactation and then decrease in parallel with the level of prolactin (Smith & Stern, 1981). A role for growth hormone (GH) has also been proposed: in the absence of

prolactin, neutralisation of GH led to reduced synthesis and uptake of fatty acids in rat mammary tissue (Barber *et al.*, 1992b).

1.2.3 Milk proteins

The milk proteins can be divided into two groups, the caseins and the milk serum or whey proteins. The caseins are the most abundant milk proteins, accounting for appproximately 60% of total milk protein, and provide essential amino acids and minerals (in the form of calcium phosphate) for the young (Bonsing & Mackinlay, 1987). The whey proteins include α-lactalbumin, β-lactoglobulin, whey acidic protein, immunoglobulins, transferrin and lactoferrin. Of the whey proteins, β-lactoglobulin is the principal protein in ruminant milk, while whey acidic protein (WAP) is the major protein in the milk of rabbits and rodents, and has recently been identified in pig milk (Simpson *et al.*, 1996). The function of WAP and β-lactoglobulin is not known, but β-lactoglobulin has structural similarities with retinol binding protein and as such may paly a role in the transport of fatty acids and vitamin A (Newcomber *et al.*, 1984) Milk protein composition is discussed in detail by Davies *et al.* (1983) and Mepham (1987).

Milk proteins are synthesised by a mechanism common to all eukaryotic proteins (reviewed by Mepham, 1987; Mercier & Gaye, 1983). Proteins which are secreted from the cell move sequentially from their site of synthesis in the rough endoplasmic reticulum through the Golgi apparatus. From the Golgi, proteins are transported by

secretory vesicles to the apical surface of the cell and released into the lumen by exocytosis (Franke *et al.*, 1976).

Milk protein synthesis is controlled by hormonal regulation of gene transcription, the stability of milk protein mRNAs and the rate of translation. Translational, transcriptional and post-transcriptional control of the major milk protein genes will be discussed in further detail in section 1.3.

1.3 The differentiated function of the mammary gland

1.3.1 Gland development and differentiation

Development of the mammary gland begins in fetal life, and at birth the gland consists of a rudimentary ductal tree (Russo & Russo, 1987). During puberty a branching pattern of ductal growth is achieved by lengthening and branching of the existing ducts. Proliferation of the actively growing structures, or terminal end buds (TEBs), at the tips of the lengthening ducts allows the ductal system to penetrate further into the mammary fat pad (Russo & Russo, 1987). The parenchyma of the differentiated mammary gland consists of ductal epithelium, alveolar epithelium and myoepithelium (Rudland & Hughes, 1989; Streuli *et al.*, 1995). Extensive ductal and lobuloalveolar development begins with each pregnancy and culminates with the onset of lactation. During pregnancy secretory tissue develops from the branching duct system. There is, in addition, extensive proliferation of secretory alveolar cells which fills the mammary fat pad with the alveolar tissue required for milk production. Development and

subsequent differentiation of the gland during pregnancy are dependent on complex interactions between a number of systemic hormones and locally produced growth factors (reviewed by Topper & Freeman, 1980; Tucker, 1994).

As they differentiate the epithelial cells become polarised: within the alveoli, cells are organised as a single layer of cells around a central lumen, into which milk is secreted. Alveolar epithelium is only occasionally contacted by myoepithelial cells and is separated from surrounding stroma by a specialised basement membrane. This basement membrane is laminin-rich and is essential for the differentiated function of the cell (Streuli *et al.*, 1995).

Epithelial cell differentiation is characterised by cell hypertrophy, accumulation of milk protein mRNAs and an increase in lipogenic and other enzymatic activity involved in milk synthesis (reviewed by Burgoyne & Wilde, 1994). Differentiation of the gland is a sequential process. Milk protein mRNAs are present by mid to late pregnancy but copious milk production does not start until parturition. In the mouse, for example, β-casein mRNA is present at mid-pregnancy and increases progressively up to and after parturition (Harris *et al.*, 1991). Conversely WAP gene expression increases predominantly after the young are born (Harris *et al.*, 1991; Pittius *et al*, 1988), while ACC and FAS activities rise in the final days of pregnancy and continue to rise until peak lactation (Shipman *et al.*, 1987). This sequential induction of epithelial cell

differentiation suggests that milk protein genes are regulated differentially within the secretory cell either by systemic hormones or by local intramammary factors.

Mammary tissue is removed after weaning of the young, in species such as mice and rodents to prevent unnecessary milk production. Without the suckling stimulus hormone levels, in particular prolactin, decline and milk accumulates within the gland. Both these events serve to decrease milk synthesis and secretion. Loss of epithelial cells occurs by programmed cell death *i.e.* apoptosis (Walker *et al.*, 1989; Strange *et al.*, 1992). In addition, extracellular matrix-degrading metalloproteinases are expressed in the gland during involution (Strange *et al.*, 1992) which are responsible for the breakdown of the basement membrane (Talhouk *et al.*, 1991, 1992). The loss of epithelial cells and the proteolytic degradation of the basement membrane leads to collapse and disintegration of the lobuloalveolar structure of the gland. The extent of cell loss during involution varies widely among species (Hurley, 1989). In rodents, the gland regresses to a state similar to that of the virgin animal (Walker *et al.*, 1989). Cell loss in ruminants is considerably less but also occurs by apoptosis (Quarrie *et al.*, 1996; Wilde *et al.*, 1997b).

1.3.2 The role of the extracellular matrix

In addition to the role of hormones, interactions between mammary epithelial cells and the extracellular matrix (ECM) play a vital role in mammary gland development and differentiation. The ECM consists of acellular material which connects cells within

tissues. Epithelial tissues, such as mammary secretory cells, contact a specialised ECM, the basement membrane. Lactogenic hormones are not sufficient to stimulate the differentiation of mammary cells and full expression of milk protein genes. The ECM is also necessary (reviewed by Streuli, 1993). Tissue specific gene expression in mammary epithelial cells is therefore dependent on both ECM and hormonal factors.

It is well established that substratum has a dramatic effect on the morphology and function of mammary epithelial cells in culture. Simplistically, such cells cultured on either plastic or fixed collagen gels do not maintain a differentiated morphology even in the presence of lactogenic hormones (Li *et al.*, 1987). Cells cultured in this way lack polarisation, the necessary secretory apparatus is underdeveloped, and expresses milk protein genes only at very low levels. Culture on floating collagen gels increases the expression of β-casein (Li *et al.*, 1987) but not that of WAP (Chen & Bissell, 1989), indicating that differentiation is not complete on this substrata. Expression of WAP is only achieved *in vitro* when cells are cultured on EHS matrix (Chen & Bissell, 1989). EHS matrix is a reconstituted basement membrane derived from the Engelbreth-Holm-Swarm murine tumour, which contains a number of basement membrane components, including collagen type IV, laminin, entactin, fibronectin and proteoglycans (Kleinman *et al.*, 1983, 1986). Culture on this substratum allows cells to polarise; the cells are columnar and contain abundant rough endoplasmic reticulum, Golgi and secretory vesicles (Aggeler *et al.*, 1991).

The basement membrane cooperates with lactogenic hormones *in vitro* to activate transcription of milk protein genes such as β-casein (Li *et al.*, 1987, Barcellos-Hoff *et al.*, 1989, Schmidhauser *et al.*, 1990, 1992). The effects of the ECM on epithelial cells are mediated by specific receptors on the basal surface of the cell which contacts the basal lamina. The basement membrane consists of a number of proteins and glycoproteins, the most important of which is laminin (Streuli, 1993). The laminins are a family of αβγ heterotrimers which have been shown to influence morphology and gene expression in cultured epithelial cells (Bissell *et al.*, 1987; Kubler *et al.*, 1991; Liu *et al.*, 1991). Specific receptor molecules termed integrins, are located in the basolateral cell membrane, and these molecules are thought to mediate signal transduction between the epithelial cell and the basement membrane. A function blocking anti-integrin antibody has recently been shown to decrease the ability of cultured cells to synthesise β-casein (Streuli *et al.*, 1991).

Further evidence for a role of the basement membrane *in vivo* has come from events which take place during involution. Following weaning, the basement membrane becomes folded (Walker *et al.*, 1989) and is ultimately removed by ECM-degrading metalloproteinases (Talhouk *et al.*, 1991, 1992). During involution the expression of ECM degrading metalloproteinases such as stromelysin, gelatinase and tissue plasminogen activator increases relative to expression of their inhibitors, tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator inhibitor (PAI-1). In the mouse, the gland returns to a state reminiscent of the virgin gland (Walker *et al.*, 1989;

Strange *et al.*, 1992) and tissue specific gene expression, such as β-casein is lost (Talhouk *et al.*, 1991). In addition, loss of cell-matrix and cell-cell contact has been shown to be associated with the onset of apoptosis (Strange *et al.*, 1992).

Recent studies have demonstrated that interactions with a basement membrane in culture protects mammary epithelia from cell death; neither collagen I or plastic substrata supports survival (Boudreau *et al.*, 1995; Pullan *et al.*, 1996). The basement membrane therefore contains a number of direct signals which are required for the differentiation and maintenance of normal mammary epithelial cells (reviewed by Ashkenas *et al.*, 1996; Streuli, 1993).

1.3.3 Regulation of milk protein gene expression

The rate of milk protein synthesis depends primarily, but not solely, on the abundance of milk protein mRNA, which increases during gestation and reaches a peak at mid-lactation. Differential accumulation of milk protein mRNAs depends on increases in both transcription rate and transcript half-life (Guyette *et al.*, 1979).

All milk protein genes sequenced so far have the canonical organisation of all eukaryotic genes, consisting of the polyadenylation signal, GT rich region signaling the end of transcription, consensus splice junction sequences, CAP site and TATA box (reviewed by Mercier & Vilotte, 1993). The activation of these genes depends on a variety of 5' and 3' regulatory sequences which have however, been only partially

identified. The current knowledge on regulation of the caseins and whey protein genes will be discussed in the following sections.

1.3.3.1 Regulation of casein gene expression

The caseins are the most abundant milk proteins and are secreted as micelles, large macromolecular aggregates (Waugh, 1971). Five caseins have been identified α_{s1}-, α_{s2}-β-, γ- and κ-casein, with κ-casein important in prevention of precipitation by stabilising micelle formation. The calcium sensitive caseins are phosphorylated and, along with κ-casein, are the primary source of amino acids, phosphate and calcium for the suckling young.

Sequence analysis of the casein gene family has revealed that although there has been considerable divergence between the members, three structural regions are highly conserved. These include the signal peptide, the casein kinase recognition site and 5' non-coding sequences (Blackburn *et al.*, 1982; Hobbs & Rosen, 1982).

Regulation of casein gene expression has been studied primarily in mammary cell culture. The synergistic action of the lactogenic hormones, principally glucocorticoid, insulin and prolactin and the extracellular matrix on β-casein gene expression is well documented (Eisenstein & Rosen, 1988; Schmidhauser *et al.*, 1990; Schmitt-Ney *et al.*, 1991).

The promoter region of the gene encoding β-casein has binding sites for numerous transcription factors which exert both positive and negative effects on β-casein gene expression (Schmitt-Ney *et al.*, 1991; Welte *et al.*, 1994). One such factor which is essential for prolactin-induced β-casein gene transcription binds to a conserved interferonγ activation (GAS)-like sequence in the promoter sequence of the gene from several species (Schmitt-Ney *et al.*, 1991). This factor is STAT5 which was initially identified as mammary gland factor (MGF). MGF (STAT5)-specific binding has also been identified in the upstream region of the bovine α_{s2}-casein gene promoter (Groenen *et al.*, 1992). The discovery of the JAK/STAT pathway has provided insight into the previously unknown signaling pathway of prolactin and its role in casein gene expression (reviewed by Hynes *et al.*, 1997).

In addition to the importance of STAT5, other elements may be involved in casein gene transcription. Important regulatory elements are located more than 800 bp upstream of the bovine β-casein promoter (Schmidhauser *et al.*, 1990). Further, a unique 160 bp enhancer element, termed BCE, lies 1.5 kb upstream of the β-casein transcription initiation site (Schmidhauser *et al.*, 1992).

1.3.3.2 Regulation of α-lactalbumin gene expression

Alpha-lactalbumin is essential for the production of lactose and thus milk (Stacey *et al.*, 1995: Stinnakre *et al.*, 1994). Expression of the α-lactalbumin gene requires insulin and prolactin and is maximal in the presence of glucocorticoid (Ono & Oka, 1980),

although high levels of glucocorticoid may inhibit α-lactalbumin expression (Funder, 1989). Progesterone also inhibits α-lactalbumin gene expression and it is the loss of progesterone at parturition, along with the increase in prolactin that allows increased α-lactalbumin protein synthesis. Indeed in marsupials, prolactin alone is responsible for expression of α-lactalbumin (Collet *et al.*, 1992). Studies in transgenic mice have indicated that efficient tissue specific expression of bovine (Vilotte *et al.*, 1989) and caprine (Soulier *et al.*, 1992) α-lactalbumin genes may be achieved using 0.4 kb of 5' flanking region and 0.34 kb of 3' flanking region (Soulier *et al.*, 1992; Stinnakre *et al.*, 1991). Multiple binding sites, including one recognised by nuclear factor-1 have also been identified by footprinting analysis of the bovine α-lactalbumin gene (Lubon & Hennighausen, 1988).

1.3.3.3 Regulation of whey acidic protein gene expression

Whey acidic protein (WAP) is expressed in high levels in the lactating mammary glands of mice, rats and rabbits (Hennighausen & Sippel, 1982; Hennighausen *et al.*, 1982; Hobbs *et al.*, 1982). WAP mRNA accumulates in late pregnancy and by mid-lactation is present at levels 1000-fold higher that that seen in early pregnancy. WAP gene expression is dependent on synergy between prolactin, glucocorticoid and insulin, cell-cell and cell-matrix interactions. WAP is poorly expressed in culture unless cells are present in three-dimensional structures similar to alveoli *in vivo* (Chen & Bissell, 1989). Deletion expression analysis using WAP-myc and WAP-CAT reporter systems in primary culture suggested that lactogenic hormone response elements are located

mainly in 2.5 kb of 5' upstream sequence (Schoenenberger *et al.*, 1990; Doppler *et al.*, 1991).

1.3.3.4 Regulation of β-lactoglobulin gene expression

β-lactoglobulin is the major protein in ruminant milk. It is expressed by mid-pregnancy, increases slowly until parturition and then increases rapidly, again reaching a peak at mid-lactation (Gaye *et al.*, 1986; Harris *et al.*, 1991). In cultures of ovine mammary cells induction of milk protein genes appears less dependent on lactogenic hormones than the caseins. Glucocorticoid and insulin in synergy with prolactin are only slightly more effective than prolactin alone in inducing β-lactoglobulin gene expression (Puissant *et al.*, 1990). In the marsupial, the increase in β-lactoglobulin mRNA appears to be dependent on prolactin alone (Collet *et al.*, 1992). CAT assay systems have demonstrated that the β-lactoglobulin promoter is sensitive to prolactin induced signals: prolactin induced CAT activity can be achieved in Chinese hamster ovary cells transfected with reporter gene constructs driven by the β-lactoglobulin 5' flanking region (Lesueur *et al.*, 1990).

The ovine β-lactoglobulin gene contains multiple binding sites within a -406 to -149 5' upstream region which are essential for high-level tissue specific gene expression (Wilmut *et al.*, 1990). These include five binding sites specific for nuclear factor-1 and three sites recognised by milk protein-binding factor (Vilotte & Soulier, 1992).

1.4 Endocrine control of lactation

1.4.1 Prolactin

Prolactin is secreted by the anterior pituitary and exerts a wide range of biological effects including its pivotal role in lactation. Prolactin is released in response to suckling or milking. Thus, the circulating concentration of the hormone is low during most of gestation and increases by greater than an order of magnitude in early lactation(Cowie *et al.*, 1980; Vines *et al.*, 1977). The milking induced prolactin surge is greatest at peak lactation and its intensity decreases as lactation progresses (Cowie *et al.*, 1980). Circulating prolactin levels change in response to season, stage of oestrus cycle, pregnancy, lactation, time of day, stress, temperature and energy intake (Vonderhaar, 1987a).

Traditionally prolactin has been considered the major hormone involved in the maintenance of lactation in rodents, but of little importance in ruminants. The ergot alkaloid bromocriptine decreases circulating prolactin and inhibits the post-milking/suckling prolactin surge (Akers *et al.*, 1981; Beck *et al.*, 1979). In lactating rats, administration of bromocriptine inhibited lactation: both litter weight gain and mammary tissue weight were decreased in these animals (Madon *et al.*, 1986; Flint *et al.*, 1992). The detrimental effect of bromocriptine was completely reversed by administration of exogenous prolactin. Depletion of prolactin in the period immediately prior to parturition inhibits lactation in cattle (Akers *et al.*, 1981) and sheep (Kann & Houdebine, 1978). The effect in cows was transient and reversed by exogenous

prolactin (Akers *et al.*, 1981). In lactating goats and cows, prolactin depletion has a much less pronounced effect on milk yield than that seen in rats and mice (Hart, 1973; Smith *et al.*, 1974). Recent studies have however suggested that prolactin is at least as important as growth hormone in the maintenance of lactation in the goat (Flint & Knight, 1997).

The prolactin receptor is a member of the cytokine receptor superfamily (Bazan, 1990). There are two forms of the receptor- a short form and a long form, differing only in their cytoplasmic domain. The long form has a cytoplasmic domain of 357-364 amino acids (Boutin *et al.*, 1988; Edery *et al.*, 1989), while the cytoplasmic domain of the short form consists of 57 amino acids (Boutin *et al.*, 1988; Davis & Linzer, 1989). Only the long form of the receptor is competent to activate milk protein gene transcription (Lesueur *et al.*, 1991). Both short and long form prolactin receptor number remains low throughout gestation, rises sharply around parturition and continues to increase throughout lactation (Hayden *et al.*, 1979; Jahn *et al.*, 1991).

The prolactin receptor is associated with Janus tyrosine kinase 2 (JAK2) (Argetsinger *et al.*, 1993; Lebrun *et al.*, 1994). Receptor dimerisation is induced by ligand (prolactin) binding (Findori & Kelly, 1995), activating JAK2 which results in phosphorylation of tyrosine residues of STATs (signal transducers and activators of transcription). The STATs are cytoplasmic transcription factors which, when phosphorylated on tyrosine, activate gene transcription (reviewed by Goffin & Kelly,

1997). These transcription factors are important prolactin binding proteins. Seven STATs have thus far been identified: 1, 2, 3, 4, 5a, 5b and 6 (Ihle, 1996). Of these STAT1, 3 and 5 have been identified as transducer molecules of the prolactin receptor (Gouilleux *et al.*, 1994; Dasilva *et al.*, 1996). STAT5 was initially identified as the nuclear transcription factor MGF (mammary gland factor) which is involved in prolactin mediated transcriptional activation of the β-casein gene (Gouilleux *et al.*, 1994; Burdon *et al.*, 1994). MGF or STAT5 is activated by phosphorylation of one tyrosine residue on STAT5 by JAK2. Tyrosine phosphorylation of STAT5 converts it from a latent factor to one which is able to bind to DNA and activate transcription (Groner & Gouilleux, 1995).

Although the JAK/STAT pathway is considered the most important signalling pathway for prolactin, other pathways may also be involved. These may include the Ras/Raf/MAPK cascade, IRS-1, PI-3 kinase, PKC or calcium ions (reviewed by Goffin & Kelly, 1997).

1.4.2 Growth hormone

Growth hormone is the major galactopoietic hormone in ruminants (Cowie *et al.*, 1980; Karg *et al.*, 1972). Serum levels of growth hormone remain low and constant for the majority of pregnancy in both rats and cows (Schalch & Reichlen, 1966; Oxender *et al.*, 1972) and increase sharply at parturition (Ingalls *et al.*, 1973).

Milk secretion in hypophysectomised lactating goats can be maintained by growth hormone alone (Cowie *et al.*, 1964) and bovine growth hormone has been shown to be a potent stimulator of mammary growth and milk production in dairy cows (reviewed by Bauman 1992). Growth hormone causes coordinated changes in protein, fat and carbohydrate metabolism in the lactating animal, directing nutrients for use in milk synthesis (Bauman & Currie, 1980; Bauman *et al.*, 1985). Growth hormone is discussed in detail by Tucker (1994) and Flint & Knight (1997).

Growth hormone has typically been thought of as having little or no role in maintenance of lactation in rodents. This view has recently been shown to be somewhat simplistic. Neutralisation of growth hormone in the rat using an anti-growth hormone antibody reduced milk yield, albeit to a lesser extent than that seen with prolactin depletion (Flint *et al.*, 1992). However a combination of both bromocriptine treatment and anti-growth hormone almost completely abolished milk secretion (Madon *et al.*, 1986; Flint *et al.*, 1992). Growth hormone replacement was only partially successful in restoring milk yield to pretreatment levels, however treatment with exogenous prolactin fully restored milk yield.

The mechanism by which growth hormone acts on the secretory alveolar cell is not understood, since a receptor for growth hormone has not yet been detected in mammary tissue (Akers, 1985). Growth hormone is therefore thought to act indirectly on the mammary gland via secondary mediators, the insulin-like growth factors (IGFs).

Insulin-like growth factors are potent mammary mitogens (Winder & Forsyth, 1986) and are involved in cellular differentiation (reviewed by Dembinski & Shui, 1987). The role of the insulin-like growth factors is discussed in section 1.5.1.

The growth hormone receptor, like the prolactin receptor is a member of the cytokine receptor family. The receptor consists of a single chain protein with a transmembrane domain. Ligand binding causes dimerisation of the GH receptor which then interacts with the JAK kinases which in turn activate STATs (reviewed by Goffin & Kelly, 1997). Like prolactin, growth hormone can activate STAT5 (Hackett *et al.*, 1995) and this activation seems dependent on receptor tyrosine phosphorylation (Sotiropoulos *et al.*, 1996).

1.4.3 Steroid hormones - oestrogen and progesterone

During pregnancy, mammary growth is stimulated by complex interactions between polypeptide and steroid hormones. The ovarian steroids, oestrogen and progesterone, are responsible for stimulation of mammary growth during both puberty and pregnancy (Lyons *et al.*, 1958; Nandi, 1958). The oestrogens promote ductal elongation, act to increase the number of proliferating cells, while decreasing the time required for cell division (Bresciani, 1971; Grahame & Bertalanffy, 1972). Progesterone stimulates ductal branching and development of lobuloalveolar tissue in the pregnant animal (Nandi, 1958). Lactogenesis is inhibited by progesterone (reviewed by Kuhn, 1977): a

reduction in plasma progesterone permits induction of milk secretion (Kuhn, 1969). The role of the sex steroid hormones is reviewed in detail by Haslam (1987).

1.4.4 Glucocorticoids

Classic endocrine ablation experiments demonstrated the requirement for glucocorticoids for lactation. Lyons *et al.* (1958) demonstrated that only prolactin and adrenal steroid were necessary for milk secretion in the endocrinectomized rat. Following hypophysectomy in the goat, glucocorticoids, thyroid hormones and growth hormone were required, in addition to prolactin to fully restore lactation in these animals (Cowie *et al.*, 1964). Serum glucocortocoid remains low throughout pregnancy, however prior to parturition the concentration of glucocorticoid increases, reaching a maximum at parturition and the initiation of lactogenesis. A role for glucocorticoid as a survival factor in mammary epithelial cells has also been proposed (Feng *et al.*, 1995). The requirement of glucocorticoids for lactation is described in detail by Tucker (1994).

1.5 Growth Factors

The capacity of the mammary gland to produce milk is determined first by systemic endocrine factors. However, there is increasing evidence that local milk borne factors, produced by the mammary epithelial cell are also involved. Some of these factors are discussed below.

1.5.1 Insulin-like growth factors

The growth promoting action of growth hormone is brought about by secondary mediators known as the insulin-like growth factors (IGFs). The insulin-like growth factors form part of a family of structurally related proteins which include insulin and relaxin. These factors are produced by most tissues within the body, are present in the circulation and may be bound to the IGF binding proteins (IGFBPs). Milk contains IGF-I and II and at least four of a possible six IGFBPs have been identified in milk (reviewed by Prosser, 1996). Additionally bovine mammary cells *in vitro* have been shown to synthesise and secrete four IGFBPs (McGrath *et al.*, 1991). IGF concentrations in milk have been shown to be highest pre-partum and early post-partum, suggesting they may play a role in development of both the mammary gland and the gut of the neonate.

Two classes of IGF receptors have been identified - type I receptors with greatest affinity for IGF-I, and type II receptors. The type I receptor is thought to be the major receptor involved in the signalling action of both IGF-I and II. IGF receptors have been found in the mammary glands of humans (Ellis *et al.*, 1994), sheep (Disenhaus *et al.*, 1988), cows and rats (Collier *et al.*, 1989). Many roles have been postulated for IGFs in the mammary gland. These include increasing growth, blood flow, milk secretion, glucose transport and possibly casein synthesis (reviewed by Prosser, 1996). *In vitro*, both IGF-I and II increase proliferation of mammary epithelial cells (Imagawa *et al.*, 1986; McGrath *et al.*, 1991): since IGF concentration is highest at the time of

maximal epithelial cell proliferation in the developing gland it is possible that this factor plays a role in determining the growth and thus subsequent output of the gland.

IGF-I may act as a survival factor for several cell types, including mammary cells (Rodriguez-Tarduchy *et al.*, 1992; Sell *et al.*, 1995). It has recently been demonstrated that IGFBP-5 is present in high concentrations in involuting mammary cells (Tonner *et al.*, 1996) suggesting that this binding protein may block IGF mediated cell survival. Prolactin, which has also been implicated in cell survival decreases IGFBP-5 production (Travers *et al.*, 1996).

1.5.2 Epidermal growth factor

Epidermal growth factor (EGF) is a small polypeptide mitogen which has been identified in the milk of mice (Beardmore & Richards, 1983), rats (Thornburg *et al.*, 1984) and humans (Cohen & Carpenter, 1975). EGF is synthesised as prepro-EGF and is subsequently cleaved to its biologically active form (reviewed by Carpenter & Wahl, 1991).

EGF receptor number is high in virgin mice, increases during early pregnancy and then decreases during lactation (Edery *et al.*, 1985). The receptor is also present in a similar pattern in the bovine (Spitzer & Grosse, 1987; Plaut, 1993) and is expressed in the ductal epithelium (Coleman *et al.*, 1988). Binding of EGF to its receptor is dependent

on synergism between estrogen and progesterone in conditions similar to that seen in pregnancy (Sheffield & Yuh, 1988).

EGF stimulates mammary development (Turkington, 1969; Tonelli & Sorof, 1980), in particular ductal and lobuloalveolar development (Vonderhaar, 1987b; Coleman *et al.*, 1988). Full development of the mouse mammary gland during pregnancy, and thus subsequent milk production, is dependent on EGF (Okamata & Oka, 1984). These observations lend further support to a role for EGF in stimulation of mammary epithelial proliferation.

In addition to its role in mammary development in the mother, milk-borne EGF has been proposed to play a role in the developing gut of the neonate. EGF receptors have been demonstrated in the gastrointestinal tract of developing rodents (Toyoda *et al.*, 1986; Gallo-Payet *et al.*, 1987). The EGF content of the small intestine in suckling rats was high (Schaudies *et al.*, 1990), however mRNA for this growth factor was not detected in the intestine or submaxillary glands of suckling rats or mice (Popliker *et al.*, 1987; Dvorak *et al.*, 1995). Milk-borne EGF may therefore be important in regulation of intestinal development in the neonatal rodent (reviewed by Koldovsky, 1996).

1.5.3 Fibroblast growth factors

The fibroblast growth factors (FGFs) are members of a larger family of heparin-binding growth factors. Such growth factors may be divided into two subclasses: class I

anionic mitogens, such as acidic brain FGF, and class II cationic mitogens (including basic pituitary FGF). Acidic and basic FGF were purified owing to their strong affinity for heparin (Macaig *et al.*, 1984). Consistent with their affinity with heparin the FGFs have been localised to, and purified from, basement membranes in a variety of tissues (Folkman *et al.*, 1988). Fibroblast growth factors stimulated cellular proliferation *in vitro* in both normal and breast cancer cells (Yang *et al.*, 1980). In addition FGF inhibited the production of casein when mammary epithelial cells are grown on collagen (Levay-Young *et al.*, 1989). Further studies confirmed that FGF inhibits the expression of milk protein genes induced by insulin, glucocorticoids and prolactin (Oka *et al.*, 1991). An FGF-like growth factor has been isolated from a human mammary tumour (Rowe *et al.*, 1986) raising the possibility that this FGFs are produced by and act on the mammary epithelial cell, influencing mammary growth (reviewed by Dembinski & Shiu, 1987).

1.5.4 Transforming growth factors

The transforming growth factors (TGFs) may be divided into 3 subclasses: TGF-α, TGF-β and TGF-γ. For the purposes of this section only TGF-α and TGF-β will be discussed.

1.5.4.1 TGF-α

Transforming growth factor-α is a 50 amino acid polypetide and is similar in size, but shares only 30 - 40% homology, with EGF. TGF-α is synthesised as a membrane

bound precursor and undergoes a number of cleavages to form the active protein (Carpenter & Wahl, 1991). TGF-α competes with EGF for the EGF receptor. As both TGF-α and EGF bind to the same receptor it is thought that these growth factors have similar, possibly identical, functions (Carpenter & Wahl, 1991). TGF-α activity has been found in mammary epithelial cells of humans, rats and mice (Liscia *et al.*, 1990; Snedeker *et al.*, 1991). Like EGF, TGF-α is a potent mitogen for mammary epithelial cells *in vitro* (Imagawa *et al.*, 1990). Stimulation of lobuloalveolar development in whole organ culture by TGF-α was greater than that seen with EGF (Vonderhaar, 1987b). Over expression of TGF-α in transgenic mice leads to mammary hyperplasia and increased susceptibility to cancer (Jhappan *et al.*, 1990; Coffey *et al.*, 1994).

1.5.4.2 TGF-β

The transforming growth factor-βs (TGF-β) are a family of hormone-like polypeptide growth factors which act as inhibitors of mammary cell growth. Three isoforms of TGF-β have been identified in mammary tissue - TGF-β1, β2 and β3. TGF-β is highest during pregnancy, decreases during lactation and rises once again in involution (Robinson *et al.*, 1991; 1993).

TGF-β exerts growth inhibitory effects on mammary epithelial cells both *in vivo* and *in vitro*. TGF-β implants in subadult virgin mice reversibly inhibit mammary ductal growth but not alveolar development (Silberstein & Daniel, 1987). Over-expression of TGF-β in transgenic mice resulted in impaired lobuloalveolar development and milk

protein production (Jhappan *et al.*, 1993). TGF-β has been shown to inhibit the growth of human mammary epithelial cells (Ethier & Van de Velde 1990). The effect of TGF-β in mammary development appears to be regulated partially by its effects on ECM (Silberstein *et al.*, 1990, 1992). In the mouse the ECM acts a reservoir for TGF-β_1 which inhibits ductal but not alveolar bud development in the tissue (Silberstein *et al.*, 1992). The role of TGF-β in mammary development is reviewed by Cunha & Hom (1996), Daniel *et al.* (1996) and Smith (1996).

In addition to its role in mammary development these growth factors also play a role in the suppression of milk synthesis and secretion. TGF-β_1 suppresses onset of lactation and subsequent production of β-casein from mouse mammary explants prepared from pregnant mice (Robinson *et al.*, 1993; Sudlow *et al.*, 1994). Protein synthesis and secretion was not affected in lactating tissue.

TGF-β exert their effects on mammary development by binding with cell surface receptors. Three major TGF-β binding proteins have been identified - types I, II and III. Type I and II receptors are serine kinases and both are required for TGF-β signal transduction (Wrana *et al.*, 1994). The type III receptor is not thought to have signalling activity (Wrana *et al.*, 1992).

1.5.5 Mammary derived growth inhibitor

Mammary derived growth inhibitor (MGDI), a peptide inhibitory factor found in the mammary gland, shows a high degree of homology to cardiac fatty acid binding protein (Bohmer *et al.*, 1988). MGDI inhibits the proliferation of both normal and transformed mammary cell lines *in vitro* (Bohmer *et al.*, 1985, 1987; Grosse & Langen, 1989) and is expressed in the differentiated mammary gland (Kurtz *et al.*, 1990). It has been suggested that the function of MGDI is to inhibit cellular proliferation in the differentiated gland. In addition, in hypophysectomised rats, induction of differentiation by lactogenic hormones increases MDGI mRNA abundance (*Kurtz et al.*, 1990). The exact role of MDGI in mammary development remains to be determined.

1.5.6 Growth factors in milk

Milk, and especially colostrum, contains high concentrations of a variety of growth factors. It is possible that, in addition to their actions within mammary tissue itself, these factors may therefore play a role in the development of the gut in the neonate (Baumrucker & Blum, 1994: Schober *et al.*, 1990; reviewed by Koldovsky, 1996). IGF-I and II have been identified in both bovine milk and colostrum (Campbell & Baumrucker, 1989; Francis *et al.*, 1988) and EGF activity has been identified in the milk of mice (Beardmore & Richards, 1983) and rats (Thornburg *et al.*, 1984). Human milk contains a wide variety of growth factors including EGF, IGFs, TGF-α and TGF-β (Koldovsky, 1989, 1994, 1995; Koldovsky & Thornburg, 1987).

1.6 Local regulation of mammary function

Locally produced growth factors play an important role in the development and regulation of mammary function (section 1.5), however, other local mechanisms have been implicated in the control of milk secretion. It has been proposed that pre-partum mammary secretions contain a locally active inhibitor of milk secretion which must be broken down before lactogenesis can be initiated (Linzell & Peaker, 1974), with a likely candidate being prostaglandin F2α (PGF2α). Goats synthesise and secrete prostaglandin F2α pre-partum, and secretion of PGF2α into the blood ceases a few days prior to parturition and is instead secreted into the milk (Maule Walker & Peaker, 1980). Removal of PGF2α by milking or metabolism of PGF2α is required to initiate copious milk secretion (Maule Walker, 1984).

Fatty acid synthesis is feedback inhibited by fatty acyl CoAs. This control mechanism could work between suckling or milking. As milk accumulates within the gland, fats also accumulate, inhibiting their own synthesis (Heesom *et al.*, 1992). At weaning milk accumulates within the gland and milk secretion is rapidly shut down. The rate at which synthesis declines is consistent with the theory of accumulation of an inhibitor in milk, and indeed rat milk inhibits fatty acid synthesis *in vitro* (Levy, 1964). FIL, the feedback inhibitor of lactation did not inhibit fatty acid synthesis *in vitro*, however coordinate inhibition of both aqueous and non-aqueous milk constituents was observed in the gland (Wilde *et al.*, 1995). These observations suggest that two possible local

mechanisms exist for regulating milk synthesis and secretion within the mammary gland.

1.7 Autocrine regulation of milk secretion

Milk production in the mammary gland is controlled by a balance of systemic and local factors. The key galactopoietic hormones in ruminants and non-ruminants are discussed in section 1.4. Local factors, produced by the mammary gland, act within the gland to regulate the rate of milk secretion. One such factor, and the one under discussion here, is FIL, the Feedback Inhibitor of Lactation (reviewed by Wilde & Peaker, 1996; Wilde *et al.*, 1997a).

1.7.1 Local regulation by milk removal

Acute regulation of milk secretion by milk removal is well documented. More frequent milk removal in both cows (Morag, 1973) and in goats increased the rate of milk secretion in the more frequently milked gland when thrice daily (Henderson *et al.*, 1983) or hourly milking (Linzell & Peaker, 1971), was compared with twice daily milking. This increase in milk yield was seen only in the more frequently milked gland. Conversely, both once daily milking (Wilde & Knight, 1989) and incomplete milking (Wilde *et al.*, 1989) reduced milk yield compared with twice daily milking. This unilateral effect was independent of systemic hormones as an increase in the rate of milk secretion was achieved in autotransplanted or denervated glands when more frequent milking was applied (Linzell & Peaker, 1971). Milk removal was required for milk yield to increase in the denervated gland, as massage of the gland without milk removal had no effect on the rate of milk secretion (Linzell & Peaker,

1971). There were two possible reasons why milk removal was essential. The first was the physical presence of stored milk. However, the increase in milk yield observed with more frequent milking was not associated with relief of pressure within the gland. When an inert sucrose solution was introduced into the gland immediately after milking, the rate of milk secretion still increased (Fleet & Peaker, 1978; Henderson & Peaker, 1984). This evidence supported the suggestion of Linzell & Peaker (1971) that the response to increased milking was, therefore, due to more frequent removal of a milk constituent.

In summary, the acute response of milk secretion rate to a change in frequency or completeness of milk removal is a local effect, seen only in the manipulated gland(s) and is related not to the physical presence (or otherwise) of milk, but to the removal of a milk constituent. These observations are compatible with the presence in milk of a chemical inhibitor whose removal regulates the rate of milk secretion.

1.7.2 Search for the chemical regulator

Goat milk fractions were tested for the presence of the putative inhibitor of milk secretion using mammary tissue explants in culture. Briefly, tissue explants were prepared from mid-pregnant rabbits and stimulated with lactogenic hormones to synthesise milk components. Milk fractions and, later, individual milk constituents were tested for their ability to inhibit casein and lactose synthesis. Initially crude milk fractions were screened; at this stage goat's milk was defatted and separated into two fractions - the caseins and the whey proteins. Inhibitory activity present in defatted milk was associated with the whey,

which reversibly inhibited the synthesis of both casein and lactose in explant culture (Wilde *et al.*, 1987a). The caseins, the most abundant milk proteins, did not affect synthesis of either casein or lactose. Further experimentation with the whey fraction indicated that the inhibitory activity was associated with a fraction of M_r 10,000 - 30,000, based on passage through or retention by ultrafiltration membranes. Intramammary injection of this crude whey fraction decreased milk secretion in both rabbits (Wilde *et al.*, 1987a) and lactating goats (Wilde *et al.*, 1988). This reduction in milk secretion *in vivo* confirmed the biological activity identified in culture bioassay.

The putative inhibitor of milk secretion was purified from the M_r 10,000 - 30,000 whey fraction. This whey fraction was resolved by FPLC anion exchange chromatography (Wilde *et al.*, 1995). Eight major protein fractions were identified based on A_{280} absorbance of eluted material, and one fraction, the third to elute, was found to inhibit both casein and lactose synthesis to the same extent as the unresolved whey fraction. No other fraction had any consistent effect. Additionally, introduction of this purified inhibitor fraction into the teat duct of lactating goats decreased milk yield (Wilde *et al.*, 1995). Again, these observations *in vivo* confirmed the biological activity identified in the bioassay system.

For protein sequencing, anion exchange-purified inhibitory protein was purified further by repeating the anion exchange chromatography. Structural analysis revealed the inhibitor to be a small, acidic glycoprotein of M_r 7,600, whose N-terminal amino acid sequence bears no resemblance to any milk protein or to any known protein (Wilde *et al.*, 1995). Based on

its novelty, and its ability to regulate the rate of milk secretion within the mammary gland, the inhibitor of milk secretion has been termed FIL, the Feedback Inhibitor of Lactation. The Feedback Inhibitor of Lactation has been found in the milk of goats (Wilde *et al.*, 1995) and cows (Addey *et al.*, 1991b), and a protein fraction with similar activity has been identified in both humans (Prentice *et al.*, 1989) and in a macropod marsupial (Hendry *et al.*, 1992).

1.7.3 Autocrine control

FIL is synthesised by the secretory epithelial cells of the mammary gland. Synthesis was demonstrated in goat mammary cells cultured on a reconstituted extracellular matrix derived from the Engelbreth-Holm-Swarm (EHS) tumour of the mouse. Culture of mammary epithelial cells on this substratum allows formation of polarised three dimensional structures which are morphologically similar to alveoli *in vivo*. These three dimensional structures, termed mammospheres, synthesise milk proteins and secrete them vectorially into a lumen formed within the structure (Barcellos-Hoff *et al.*, 1989). In this culture system, FIL was secreted apically, along with other milk constituents, into the mammosphere lumen (Wilde *et al.*, 1995). Synthesis of FIL by the cells on which it acts suggests that feedback inhibition of milk secretion is an autocrine process.

For FIL's action on the rate of milk secretion to be an autocrine process, a receptor for FIL must exist on the apical side of the mammary epithelial cell. While such a receptor has not yet been isolated, there is increasing evidence that it exists. As discussed previously (section

1.7.2), injection of FIL via the teat canal decreases the rate of milk secretion (Wilde *et al.*, 1995). Additionally, auto-immunisation of lactating goats against their own inhibitory protein was found to stimulate milk secretion (Wilde *et al.*, 1996). An increase in milk secretion was only observed when antibodies against FIL were present in milk, and not when they were present in the bloodstream (Wilde *et al.*, 1996). These observations infer that FIL acts after secretion and is susceptible to immunoneutralisation only when antibody is present in milk.

1.7.4 Influence of gland anatomy

Differences in gland anatomy affect each gland's susceptibility to autocrine inhibition by FIL. Studies have demonstrated that the response to milking frequency is dependent on the site of milk storage within the gland: animals which store a large proportion of their milk in the alveoli (the site of autocrine inhibition) are more susceptible to autocrine feedback, and accordingly show a greater response to more frequent milk removal (Knight *et al.*, 1989). Conversely, animals with large cisternal storage are more tolerant of once daily milking and less responsive to thrice daily milking (Dewhurst & Knight, 1992). These observations are consistent with the theory that FIL acts via the apical surface of the mammary secretory cell to regulate the rate of milk secretion (Henderson & Peaker, 1984; Wilde *et al.*, 1995).

1.7.5 FIL's mechanism of action

The way in which FIL regulates milk synthesis and secretion is as yet unknown, but evidence suggests that FIL reduces milk secretion as a direct consequence of inhibition of

the secretory pathway. Pulse chase experiments in lactating mouse mammary acini demonstrated that FIL inhibited secretion of pre-formed [^{35}S] labelled protein (Rennison *et al.*, 1993). FIL's inability to inhibit ionomycin-stimulated secretion also suggests that FIL acts at an early stage of the secretory process (Rennison *et al.*, 1993). Histological examination of cells exposed to FIL also indicated its effects were exerted early in the secretory process. Within 1 h of addition of FIL the Golgi apparatus became dispersed throughout the cytoplasm; however within 1 - 2 h of removing FIL, cells resumed their normal appearance (Rennison *et al.*, 1993). Blockade of the secretory process may in turn account for FIL's effect on protein synthesis. It is thought that FIL may act in a manner similar to that of the drug Brefeldin A. This fungal drug is a known inhibitor of endoplasmic reticulum to Golgi secretory protein transport (Misumi *et al.*, 1986; Lippincott-Schwartz *et al.*, 1989) and inhibits both secretion and synthesis of milk protein in murine acini culture (Rennison *et al.*, 1993). Due to its effects on membrane trafficking FIL may also have longer term effects on hormone receptor number and distribution (McKinnon *et al.*,1988; Bennett *et al.*, 1990), with the result that this protein may also be a regulator of epithelial cell differentiation within the tissue. These longer term effects on differentiation are discussed in more detail in section 1.7.6.

1.7.6 Local regulation of cell differentiation

The local increase in milk yield observed in response to increased milk removal from the gland is maintained for as long as frequent milking is applied and is sustained by developmental responses in the more frequently milked gland. Ten days of more frequent

milking elicits a significant increase in secretory cell differentiation as measured by the activities of several key enzymes involved in milk synthesis, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and galactosyltransferase (Wilde *et al.*, 1987b). In addition, prolonged frequent milking is accompanied by an increase in the number of secretory cells in the more frequently milked gland (Knight *et al.*, 1990; Wilde *et al.*, 1987b). Conversely, reduced milking frequency or incomplete removal of milk from the gland results in a decrease in the degree of secretory cell differentiation, as indicated by decreased milk yield and lowered activities of key enzymes involved in milk synthesis (Wilde *et al.*, 1989; Wilde & Knight, 1990).

More frequent milking has also been observed to have an effect on mammary gene expression. After 23 weeks of more frequent milking, higher activities of two key enzymes, FAS and ACC, were associated with an increase in abundance of messenger RNA (mRNA) for these proteins, indicating that the effects of more frequent milk removal are exerted at the level of gene expression (Travers & Barber, 1993; Wilde *et al.*, 1990). This increase in abundance of FAS and ACC mRNA may be due to either an increase in transcription rate (Guyette *et al.*, 1979), an increase in mRNA stabilisation (Eisenstein & Rosen, 1989) or a combination of both. These changes are hormone dependent, and in particular dependent on galactopoietic hormones such as prolactin (Matusik & Rosen, 1978; Teyssot & Houdebine, 1980). How these differential changes in gene expression are achieved is not clear but one possibility is that this is a consequence of local modulation of the cells' sensitivity to circulating hormones by up or down-regulation of cell surface hormone

receptors (Wilde *et al.*, 1990). Previously, increases in milking frequency have been shown to be associated with increases in the number of prolactin receptors in the more frequently milked gland (McKinnon *et al.*, 1988). A whey fraction containing FIL has been found to down-regulate prolactin receptors in primary cell culture (Bennett *et al.*, 1990), and this effect has recently been reproduced using purified FIL protein (Bennett, 1993). Differential changes in prolactin sensitivity elicited by FIL may, therefore, be competent to elicit unilateral changes in secretory cell differentiation and local modulation of milk protein gene expression.

1.8 Aims of this thesis

The response of the mammary gland to changes in frequency or completeness of milk removal is mediated by FIL, the Feedback Inhibitor of Lactation. This milk constituent is synthesised by the mammary epithelial cell: once secreted it feeds back on the secretory cell to inhibit milk production. Alterations in the frequency and efficiency of milk removal are accompanied by changes in the number of prolactin receptors (and perhaps other hormone receptors) present in the manipulated gland. Studies in primary cell culture demonstrate that FIL may be competent to modulate the cells' sensitivity to circulating prolactin. In view of FIL's ability to down-regulate prolactin receptors, it is possible that this protein is a regulator of mammary differentiation. An objective of this project was to determine if there was indeed a causal link between FIL action and mammary gene expression. This thesis attempts to answer two questions: is frequency of milking able to regulate milk protein gene expression, and if so, is this effect due to FIL, the autocrine regulator of milk secretion?

The developmental effects of frequent milking have been characterised incompletely. Previous studies have measured only the mRNA abundance and activity of the lipogenic enzymes fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). However FAS, ACC and milk protein genes are not necessarily coordinately regulated. Objective one was therefore to determine whether an increase in milking frequency is also competent to up-regulate milk protein gene expression.

FIL has been characterized by its effects on product secretion. Previous studies indicate that FIL modulates the sensitivity of the epithelial cell to circulating hormones. It is plausible that FIL may therefore influence gene expression, since this is primarily regulated by systemic hormones such as prolactin. No causative link has, however, been established between FIL and the developmental changes which accommodate each gland's response to a long-term change in milking frequency or efficiency. The second objective of this study was to determine whether FIL may itself be responsible for the developmental response of the gland to alterations in milking frequency.

If FIL is able to regulate mammary cell differentiation in primary cell cultures, it nevertheless remains to be determined if the protein's concentration changes *in vivo* in a manner consistent with acute regulation of milk secretion or long term regulation of tissue development. A key determinant of FIL concentration in milk is likely to be the level of FIL gene expression in mammary tissue, but as yet nothing is known about the gene or its regulation. Therefore the third element of the project was to clone the FIL gene in goat

mammary tissue and measure FIL gene expression *in vivo* and in primary cell culture. In particular we wished to determine if FIL is coordinately regulated with other milk proteins and if, in addition, FIL gene expression is itself under autocrine control.

Chapter Two

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals

General laboratory chemicals were supplied by Sigma (Poole, UK), BDH (Poole, UK) and Boehringer Mannheim (Lewes, UK) unless otherwise indicated. Restriction and modifying enzymes were supplied by Boehringer Mannheim, Gibco BRL (Paisley, UK), Pharmacia (St. Albans, UK) and Promega (Southampton, UK).

For procedures involving the manipulation of nucleic acids, all chemicals were molecular biology grade, and water was double distilled, deionised molecular biology grade from BDH. Cell culture media, serum, trypsin and Versene were purchased from Gibco BRL, hormones, trypsin inhibitor and antibiotics were obtained from Sigma. Collagenase (Worthington type III, 151 U/mg) was supplied by Lorne Laboratories (Reading, UK), hyaluronidase (from ovine testes; 1000 U/mg) was from Boehringer Mannheim and Dispase was supplied by Universal Biologicals (London, UK).

Engelbreth-Holm-Swarm tumour raised in mice (Kleinman *et al.*, 1986) was supplied by D Blatchford at HRI. The ovine α_{s1}-casein cDNA probe was a kind gift from Dr J C Mercier, INRA, Jouy-en-Josas, France and the caprine α-lactalbumin and rat 28S RNA cDNA probes were provided by Drs M Barber and M Travers at HRI. The ovine β-lactoglobulin cDNA was supplied by Pharmaceutical Proteins Limited (Roslin, UK). Bovine α-FIL antibody was provided by F Campbell, HRI.

2.1.2 Sterilisation of media and equipment

All equipment and solutions for manipulation of nucleic acids were rendered nuclease free by autoclaving at 121°C and 15 psi for 20 min. Equipment and media for cell culture were sterilised as above. Media containing thermolabile components, such as amino acids, were filter sterilised by passage through a 0.2 μm filter (Gelman Sciences, Southampton, UK).

2.1.3 Radiochemicals

[α-^{32}P]dCTP, [γ-^{32}P]ATP and [α-^{35}S]ATP were from NEN Dupont (Stevenage, UK) or ICN Flow (Irvine, UK). The activity of samples was determined by Cerenkov counting (50% efficiency). L-[^{35}S]methionine (cell labelling grade) was also obtained from NEN Dupont.

2.1.4 Animals

British Saanen goats were from the Institute herd and were routinely milked twice daily at 0800h and 1600h. Individual gland milk yield and time of milking were recorded. Animals were fed 1.5-1.8 kg concentrates daily (Goat mix No. 1, Edinburgh School of Agriculture, Edinburgh, UK), with half the ration given at each milking. Hay and water were available *ad libitum.*

2.2 PREPARATION AND CHARACTERISATION OF RNA

2.2.1 Mammary biopsy

Biopsies of mammary parenchyma were obtained under sodium pentobarbitone anaesthesia as described by Knight & Peaker (1984). Tissue was taken from both glands of each goat 9 days after parturition (pre-treatment, frequent milking study only), and following six weeks of treatment (post-treatment, all groups). Biopsy samples were rapidly trimmed to leave only undamaged parenchyma and immediately placed in liquid nitrogen. Further tissue samples were obtained under terminal anaesthesia.

2.2.2 Isolation of total RNA

Total RNA was prepared using a modification of the guanidium isothiocyanate/caesium chloride method as described by Chirgwin *et al.* (1979). Briefly, the tissue (typically 0.5 - 1.0 g) was ground to a fine powder in liquid nitrogen and placed in GIT (50% (w/v) guanidium isothiocyanate, 0.05 M Tris-HCl pH 7.5, 0.01 M EDTA pH 7.5, 5% (w/v) Sarkosyl (BDH), 1% (v/v) β-mercaptoethanol). The DNA was sheared by sequential passage through 21 and 23 gauge needles, and insoluble calcium phosphate was removed from the preparation by centrifugation (3,000*g*, 20°C, 10 min). The supernatant was layered on a 1.2 ml caesium chloride cushion (5.7 M CsC1 in 0.1 M EDTA, pH 7.5), and centrifuged at 149,000*g*, 20°C for 18 h. The RNA pellet was resuspended in 1 ml of water and extracted with an equal volume of chloroform:butanol (4:1 v/v). The organic layer was back-extracted with an equal volume of water, the aqueous layers pooled and the RNA precipitated at -20°C, with

the addition of 0.1 volumes of 3 M sodium acetate pH 6.0 and 2.5 volumes of absolute alcohol. RNA was subsequently recovered by centrifugation (12,000*g*, 4°C, 20 min), lyophilised, resuspended in 200 µl water and stored at -70°C. RNA concentration was determined spectrophotometrically (1 $0D_{260}$ = 40µg RNA). An A_{260}:A_{280} value in the range 1.8 - 2.0 indicated the RNA preparation was free of protein and DNA contamination.

2.2.3 Electrophoresis of RNA

RNA was separated on the basis of size by electrophoresis in a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde in MOPS buffer (0.2 M morpholinopropane sulphonic acid, 80 mM sodium acetate, 10 mM EDTA pH 7.0) with 0.5 µg/ml ethidium bromide. The RNA was resolved for 3 h at approximately 50 volts. Electrophoresed RNA was visualised on a short-wave UV transilluminator and photographed using a Polaroid land camera and Polaroid type 665 film.

2.2.4 Northern blotting

A known concentration of RNA (5-40 µg) was resolved as described in section 2.2.3 and transferred to nylon membrane (Biotrans, ICN, Irvine, California) by capillary blotting in 10x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for a minimum of 12 h, as described by Davis *et al.* (1986). Once transfer was complete the RNA was bound to the nylon membrane using a UV crosslinker (Spectrolinker, Spectronics Corp., New York, USA) according to the manufacturer's instructions.

2.2.5 [α-^{32}P] labelling of double-stranded DNA in the presence of melted agarose

DNA was resolved on a low-melting-temperature agarose gel (1% w/v) in Tris-acetate electrophoresis buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, pH 7.2). The band of interest was excised, and the DNA labelled with [α-^{32}P] dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Feinberg & Vogelstein, 1984). The labelling reaction, comprising 12.5 ng of DNA, 2.5 μl of oligo labelling buffer (OLB), 0.5 μl of 10 mg/ml BSA, 12.5 μCi [α-^{32}P]dCTP at 3000 Ci/mmol and 5 units of Klenow fragment, was incubated at 22°C for 2.5 - 12 h. OLB contained 250 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM each of dATP, dCTP, dGTP and dTTP, 1 M Hepes pH 6.6 and 1 mg/ml hexadeoxyribonucleotides (Pharmacia). Unincorporated nucleotides were removed by gel filtration through a 1 ml column of Sephadex G50 (Pharmacia) in NE (50 mM NaCl, 1 mM EDTA, pH 7.0). Probes were labelled to high specific activity, typically $1x10^6$ counts per ml of hybridisation buffer.

2.2.6 Hybridisation conditions

Northern blots were hybridised at 42°C, in standard hybridisation buffer containing 50% (v/v) formamide, to either α-lactalbumin or α_{s1}-casein cDNA probes, labelled as described in section 2.2.5. Nylon membranes were incubated in pre-hybridisation buffer for a minimum of 4 h. Formamide pre-hybridisation buffer contained 50% (v/v) deionised formamide, 5x SSC (prepared from 20x stock: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% (w/v) SDS, 200 μg/ml denatured salmon sperm DNA, 5x Denhardt's (prepared from a 50x stock containing 1% (w/v) Ficoll (Pharmacia), 1%

(w/v) polyvinyl pyrrolidone, 1% (w/v) bovine serum albumin) and 0.05% (w/v) pyrophosphate tetrasodium salt. The labelled probe was denatured by heating to 100°C for 5 min and hybridised to membrane bound RNA in pre-hybridisation buffer for 16 - 24 h at 42°C. Membranes were washed in 2x SSC/0.1% SDS (w/v) three times at room temperature, followed by a final wash in 0.2x SSC/0.1% (w/v) SDS at 50 - 60°C.

2.2.7 Autoradiography

Nylon membranes were heat-sealed in plastic bags and exposed to X-ray film (Genetic Research Instrumentation Ltd.) with intensifying screens at -70°C. Films were developed after exposure times of 2 h to 14 days. The resulting autoradiographs were scanned using a BioRad Densitometer and 1D software (BioRad Laboratories, Herts., UK).

2.2.8 DNA assay

The DNA content of samples was assessed by a fluorimetric method as described by Labarca & Paigen (1980) using calf thymus DNA (1 mg/ml stock) as the DNA standard. Cell lysates were prepared by sonication (Kontes KT50 cell disrupter, setting 20, 15 s) in DNA assay buffer (2 M NaCl, 0.1 M NaH_2PO_4, pH 7.4) and an appropriate volume removed for assay. Test samples were compared with DNA standards within a range of 1-10 μg. All assay volumes were made up to 1 ml with DNA assay buffer and 1 ml fluorimetric reagent was added. The fluorimetric reagent, bisbenzimidazole, was prepared as a 1 mg/ml stock in water and diluted to 3 μg/ml with DNA assay buffer immediately prior to use. After 20 min at room temperature, fluorescence was

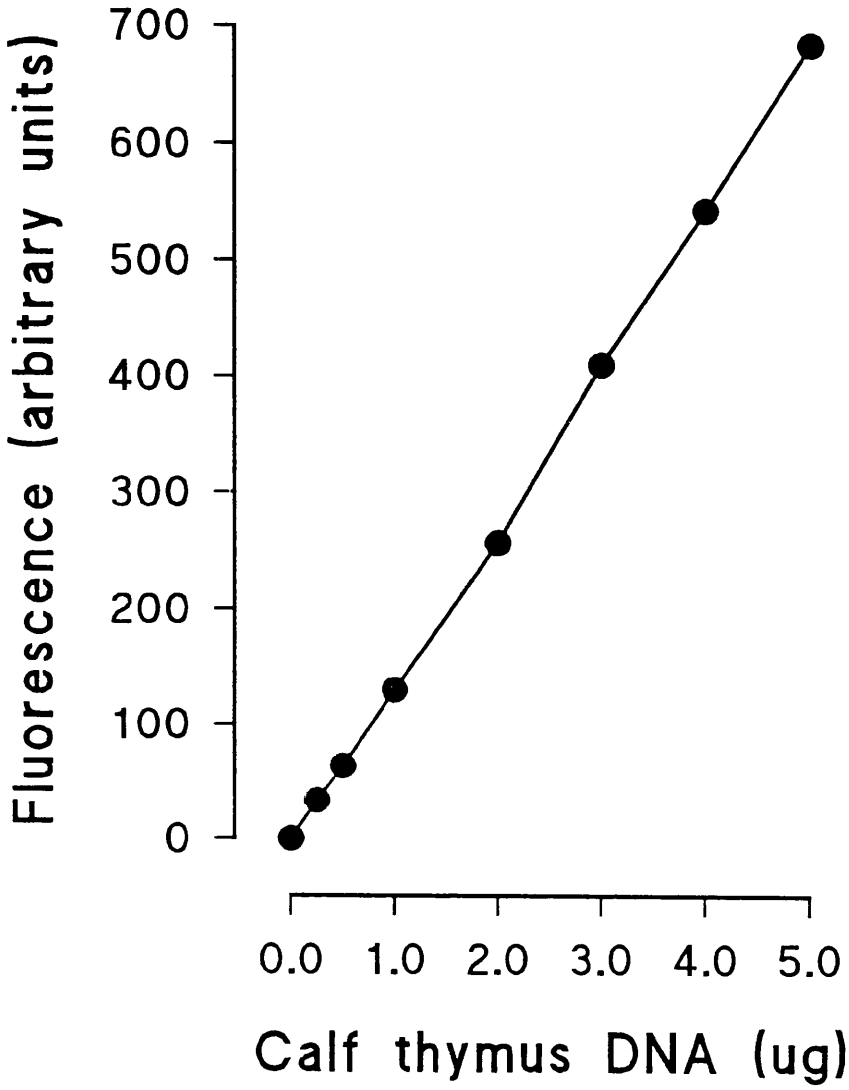


Figure 2.1 DNA Assay standard curve. The DNA content of samples was assessed by a fluorimetric method as described in section 2.2.8.

measured on a Hoeffer TK100 fluorimeter at 50% sensitivity. A typical standard curve is shown in figure 2.1.

2.3 PROTEIN PURIFICATION

2.3.1 Preparation of a M_r 6,000 - 30,000 goat whey fraction

A M_r 6,000 - 30,000 fraction was prepared essentially as described by Wilde *et. al.* (1987). 1 litre of goat's milk was collected at the morning milking and treated with protease inhibitors (2g ε-amino n-caproic acid and 0.348g phenylmethylsulphonyl fluoride in 10 ml ethanol) Milk was defatted by centrifugation (2,200*g*, 10°C, 10 min) and filtration through glass wool. The defatted milk was processed further by centrifugation (30,000*g*, 10°C, 2 h and the clear supernatant constituting the whey fraction was filtered through a 0.2 μm filter (Whatman International, Maidstone, UK), followed by ultrafiltration with filters with a molecular weight cut-off of M_r 30,000 (Minitan filtration system, Millipore, Bedford, USA). The filtrate was dialysed against water for 24 h at 4°C using dialysis tubing with a nominal molecular weight cut-off of M_r 6,000 - 8,000 (Spectrapor, Pierce & Warriner, Chester, UK), lyophilised and stored at -20°C.

2.3.2 Isolation of the feedback inhibitor of lactation (FIL)

The M_r 6,000 - 30,000 fraction of caprine whey proteins was resolved by anion exchange chromatography using a Mono Q HR 10/10 column (FPLC System, Pharmacia, Uppsala, Sweden), 10 mM bisTris propane pH 7.0 and a 0 - 1.0 M sodium acetate gradient (figure 2.2) as described by Wilde *et al.* (1995). The protein fraction

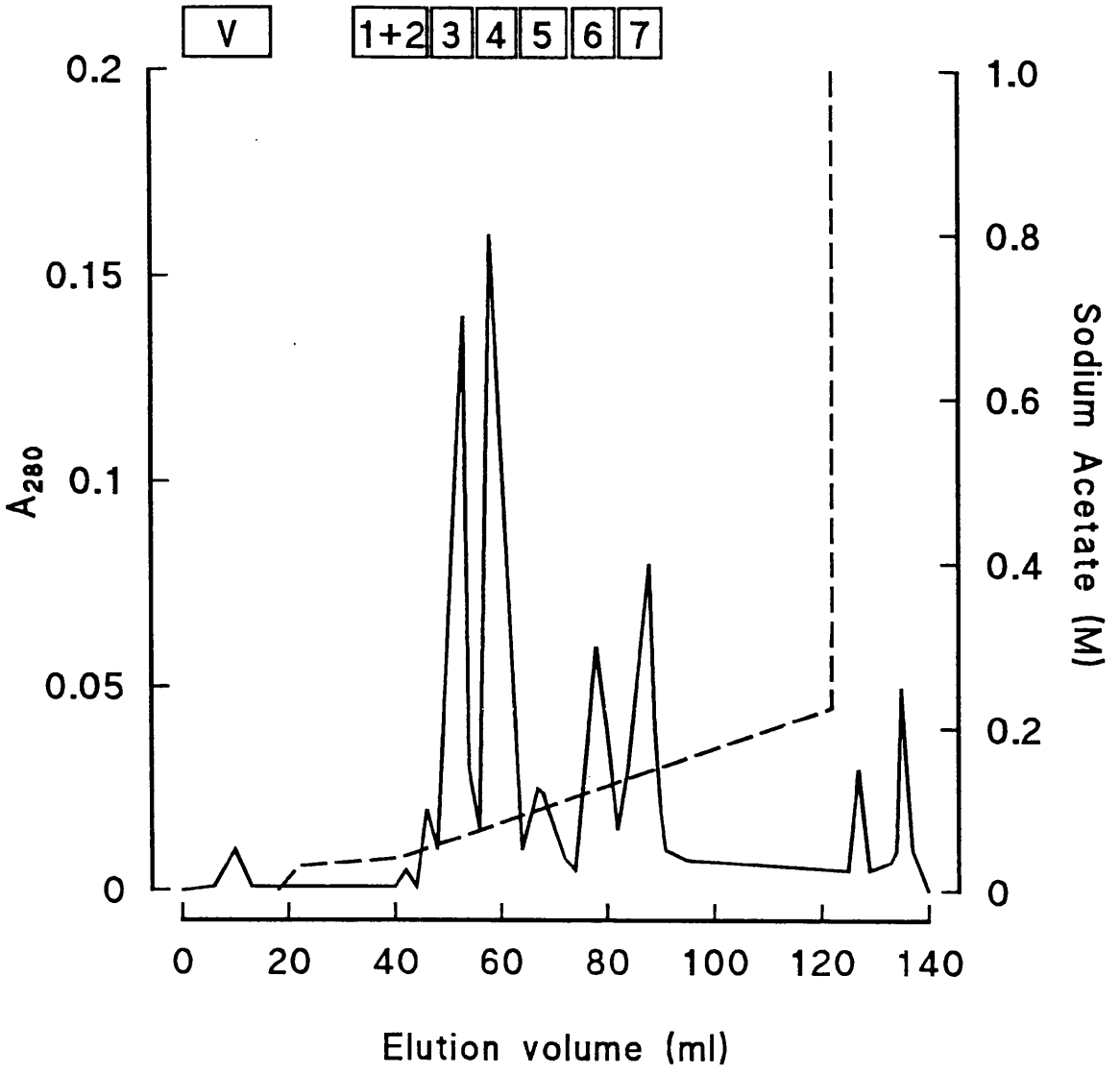


Figure 2.2 Resolution of a M_r 6,000 - 30,000 goat whey fraction. The whey fraction was resolved by anion exchange chromatography, using a Mono Q HR 10/10 column (FPLC System, Pharmacia), 10 mM bistris propane, pH 7.0 and a 0-1.0 M sodium acetate gradient. FIL was collected as the third resolved fraction (3). V, void volume containing material not bound by the column.

was dialysed against water for 24 h at 4°C, lyophilised and stored at - 20°C. Lyophilised FIL was reconstituted in 10 mM Hepes, pH 7.4 and used at a protein concentration of 8μg/ml.

2.3.3 Isolation of total whey proteins

Goat's milk was treated with protease inhibitors and defatted as described in section 2.3.1. Defatted milk was centrifuged (30,000*g*, 20°C, 2 h) and the clear supernatant constituting the whey fraction filtered through a 0.2 μm filter (Whatman). The whey fraction was dialysed against water for 24 h at 4°C using dialysis tubing with a nominal molecular weight cut-off of M_r 6,000 - 8,000 (Spectrapor) lyophilised and stored at -20°C. Lyophilised whey was reconstituted in 10 mM Hepes, pH 7.4 and used at a protein concentration of 8 μg/ml.

2.3.4 Protein assay

The protein content of samples was assessed as described by Bradford (1976), using bovine serum albumin (0.1mg/ml) as the protein standard. Dye binding reactions were carried out in microtitration plates, with standards (0-8 μg) and unknowns in a final volume of 100 μl. 240 μl of fourfold diluted Bradford reagent (BioRad Laboratories Ltd) was added to each sample well and colour formation measured at 620 nm. A standard curve is shown in figure 2.3.

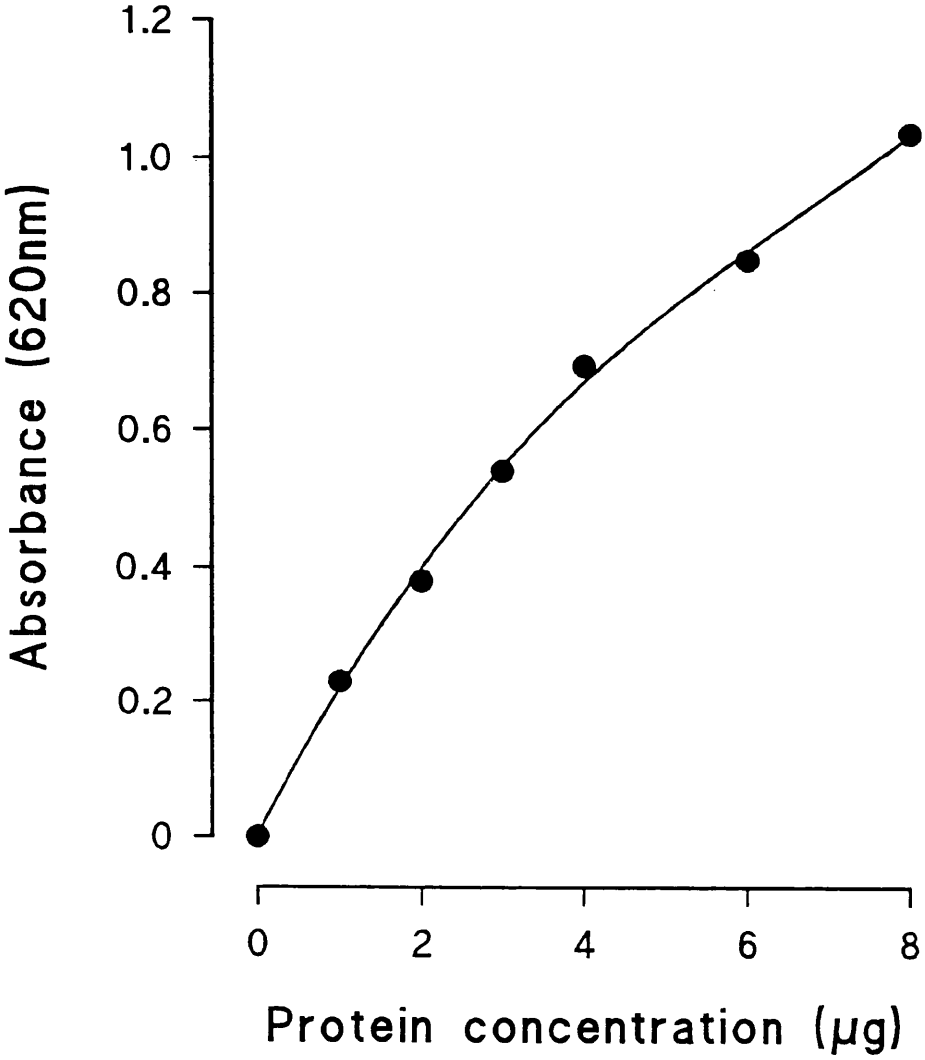


Figure 2.3 Protein Assay standard curve. The protein content of samples was assessed as described in section 2.3.4.

2.4 CELL CULTURE

2.4.1 Preparation of goat mammary epithelial cells

Goat mammary epithelial cells were prepared essentially as described by Hansen & Knudsen (1991). Mammary tissue was obtained aseptically from non-lactating, late pregnant goats (day 108 of pregnancy). Tissue was immediately placed in Hanks balanced salt solution (HBSS), pH 7.4. Connective tissue and fat were removed by dissection in a sterile cabinet. Tissue was then cut into pieces with an approximate size of 0.5 cm^2. Tissue (30g) was then placed in digestion medium, HBSS pH 7.4, containing 1.2 mg/ml collagenase, 0.5 mg/ml hyaluronidase, 1 mg/ml glucose, 0.12 mg/ml penicillin, 0.1 mg/ml kanamycin, 2.5 μg/ml amphotericin B, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 40 mg/ml bovine serum albumin, 1x MEAA (Minimum Essential Amino Acid solution, Gibco BRL), 2.5 μg/ml $MgSO_4$, 2.2 μg/ml $CaCl_2$ and 2 mM L-glutamine. Tissue pieces were injected with digestion medium until distended and incubated in an orbital incubator (120 rpm) for 2 h at 37°C. Single cells and cell debris were removed by centrifugation (80*g*, 7 min) and discarded. Undigested tissue was minced to a pulp with curved scissors and incubated for at least 2 h in digestion medium. Cells were subsequently collected by centrifugation (80*g*, 7 min), and undigested tissue removed by filtration through 150 μm nylon mesh. Cells were washed in HBSS containing 100 μg/ml trypsin inhibitor, 20 μg/ml DNAse I, 0.12 mg/ml penicillin, 0.1 mg/ml kanamycin, 2.5 μg/ml amphotericin B, 1 μg/ml hydrocortisone, 5 μg/ml insulin, 1x MEAA, 2.5 μg/ml $MgSO_4$, 2.2 μg/ml $CaCl_2$, 2 mM L-glutamine and 5.4 mM Hepes, pH 7.4. Cells were fractionated by Percoll density gradient centrifugation to obtain an epithelial cell-rich fraction. This cell fraction was

cultured on either EHS matrix or plastic as described in sections 2.4.7 and 2.4.8, or cryopreserved in foetal calf serum (FCS) containing 10% (v/v) DMSO at a density of 1 x 10^7 cells/ml.

2.4.2 Recovery of primary cells from liquid nitrogen storage

Cells were removed from liquid nitrogen and thawed at 37°C. The cells were transferred to a sterile plastic tube and 15 ml of culture medium added, dropwise. Cells were harvested by centrifugation (80*g*, 4°C, 5 min), the supernatant discarded and the cell pellet resuspended in culture medium.

2.4.3 Estimation of cell number

Cell viability was assessed by exclusion of the dye trypan blue (Freshney, 1983) and cell number was determined using a Neubauer counting chamber.

2.4.4 Preparation of culture medium

Culture medium was prepared using Hams F12 and Medium 199 (1:1 v/v). Medium contained 2 mM sodium acetate, 10mM Hepes, pH 7.4, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 3 μg/ml prolactin, 2.5 μg/ml transferrin, 200 units penicillin, 200 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Culture medium for cells grown on plastic was as above without transferrin and prolactin, but with the addition of 10 ng/ml epidermal growth factor, horse serum (20%) and FCS (5%).

2.4.5 Preparation of cell culture reagents

Cortisol (100 μg/ml) was prepared by dissolving 1 mg of hydrocortisone-21-acetate in 1 ml of absolute alcohol and diluting to 10 ml with distilled water. Prolactin (100 μg/ml) was prepared by dissolving 1 mg of prolactin in 250 μl of 270 mM Hepes, pH 8.0 before dilution to 10 ml with distilled water. Insulin (100 μg/ml) was prepared by dissolving 1 mg of insulin in 1 ml of distilled water with the addition of 10 μl of 0.34 M NaOH, before dilution to 10 ml with distilled water. Transferrin (1 mg/ml) was prepared by dissolving 1 mg of transferrin in 1 ml distilled water and epidermal growth factor (10 μg/ml) by dissolving 100 μg of epidermal growth factor in 10 ml of 0.154 M NaCl.

2.4.6 Cell culture conditions

Cells were cultured in 5% CO_2/ 95% air at 37°C and 100% humidity.

2.4.7 Cell culture on plastic

Cells were plated at a density of 8 x 10^5 per 35 mm well in 4 ml of medium. Horse serum (20%) and FCS (5%) were present throughout. The medium was replaced every two days. Cells were recovered first by incubation with Versene (5 min, 37°C), followed by treatment with trypsin (1 ml per well) for 5 - 15 min at 37°C. Harvested cells were pelleted by centrifugation (12,000*g*, 4°C, 5 min), snap frozen and stored in liquid nitrogen. Alternatively, cells were immediately re-plated on EHS matrix (section 2.4.8).

2.4.8 Cell culture on EHS matrix

Culture wells (35 mm diameter) were coated with 0.25 ml of ice-cold EHS matrix which was allowed to gel at 37°C. Cells were plated at densities of 8×10^5 or 1.6×10^6 cells per well in 4 ml of culture medium. Horse serum (20%) and FCS (5%) were initially present in the culture medium to aid cell attachment to the matrix. This medium was replaced with serum free medium after 24 h, and changed daily thereafter. Cells were recovered by treatment with Dispase (1 ml per well) for 15 min at 37°C. Harvested cells were pelleted by centrifugation (12,000*g*, 4°C, 5 min), snap frozen and stored in liquid nitrogen.

Cells were cultured on EHS matrix as described for 6 days. Cultured cells were exposed to FIL (8 μg/ml), total whey protein (8 μg/ml), brefeldin A (5 μg/ml) or actinomycin D (2.5 μg/ml) for the last 4, 24 or 72 h of culture. Exact conditions are discussed further in section 4.2.

2.4.9 RNA isolation from cultured cells and northern analysis

Total RNA was prepared from cultured cells using an Ultraspec II total RNA isolation kit (Ames Biotech, Witney, UK), according to the manufacturer's instructions. Electrophoresis, northern blotting and hybridisation with α-lactalbumin and α_{s1}-casein cDNA probes were performed as described previously (sections 2.2.3 - 2.2.7). The rat 28S RNA and the ovine β-lactoglobulin cDNA probes were labelled using the Prime-a-gene labeling system (Promega) according to manufacturer's instructions.

2.4.10 Protein synthesis and secretion in culture

Protein synthesis and secretion were determined after incorporation of L-[^{35}S] methionine using a continuous labelling protocol. Cells were incubated with 150 μCi/ml L-[^{35}S] methionine (cell labelling grade, specific activity >1000 μCi/mmol) for 4 h on day 6 of culture after various periods of incubation (24 or 72 h) with or without 8 μg/ml goat FIL or 8 μg/ml total whey protein. Prior to radiolabelling, cells were cultured for 1 h in methionine-free Minimal Essential Medium containing hormones as discussed in section 2.4.4. After labelling, the culture medium was removed and each well was washed twice in Hanks balanced salt solution (HBSS), pH 7.4. Cells were then incubated in HBSS containing 2.5 mM EGTA for 20 min at 37°C. The EGTA extract was removed and cells were recovered by treatment with Dispase (1 ml per well) for 15 min at 37°C.

2.4.11 Measurement of [^{35}S] - labelled protein

Medium samples and EGTA extracts were assayed for incorporation of radiolabel by TCA precipitation (Hurley *et al.*, 1994). Cell lysates were prepared by sonication (Kontes KT50 cell disrupter, setting 20, 15 s) in DNA assay buffer (2 M NaCl, 0.1 M NaH_2PO_4, pH 7.4). Total radiolabel incorporated in cellular protein, proteins secreted into medium and EGTA extracts was measured by precipitation at 4°C with 10% (w/v) TCA. The precipitate was collected by centrifugation (12,000*g*, 4°C, 5 min) washed twice with 1% (w/v) TCA and dissolved in 200 μl of 50 mM Tris-HCl pH 7.5 for counting of radioactivity. The DNA content of the cell lysates was assessed by a fluorimetric method as described in section 2.2.8.

2.5 ANTIBODY PREPARATION

2.5.1 Preparation of bovine α-FIL antibody

Bovine FIL was purified by anion exchange chromatography from a M_r 6,000 - 30,000 fraction of bovine whey proteins (Addey *et al.*, 1991b). Freeze-dried FIL was dissolved in 0.1 M sodium phosphate buffer, pH 6.8, and conjugated to bovine albumin by incubating equal amounts of the two proteins with glutaraldehyde as described by Wilde *et al.* (1996b), and used to immunise a female New Zealand white rabbit. A mixture of Pluronic L121 (ICI Chemicals, Runcorn, Cheshire, UK), squalene and Tween 80 (12.5:25:1, v/v) was used as an adjuvant (Wilde *et al.*, 1996b). Two further injections at four weekly intervals were used to boost the antibody titre and the rabbit was bled 10 days - 2 weeks after each immunisation.

2.5.2 Purification of bovine α-FIL antibody

IgG was isolated from serum by ammonium sulphate precipitation (Harlow & Lane, 1988). The IgG fraction was purified by affinity chromatography on Protein A agarose beads using a modification of the method of Ey *et al.* (1978), as described by Harlow & Lane (1988). Briefly, 2 ml of the IgG fraction was loaded onto a 2 ml column of Protein A agarose. Following sequential washes with 20 ml of 100 mM Tris-HCl pH 8.0 and 20 ml of 10 mM Tris-HCl pH 8.0, the purified IgG fraction was eluted from the column with 100 mM glycine-HCl, pH 3.0. The eluate was collected in 500 µl fractions, each fraction was assayed for protein content (section 2.3.3) and the protein-containing fractions were pooled. Purified antibody was stored in aliquots at -20°C.

2.5.3 SDS-PAGE under reducing conditions

Protein samples were analysed by SDS-polyacrylamide gel electrophoresis using the SDS-discontinuous buffer system of Laemmli (1970). 12% (w/v) polyacrylamide slab gels were prepared in 37 mM Tris-HCl pH 8.8 containing 0.1% (w/v) SDS. Gels were polymerised by the addition of 0.05% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. Samples were diluted 1:1 with 2x concentrated electrophoresis sample buffer (1.25 M Tris-HCl pH 6.8, 4% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.002% (w/v) Bromophenol blue) and heated at 100°C for 3 min prior to loading on the gel. Electrophoresis was carried out at 35 mA for 1 h in running buffer containing 0.25 M Tris-HCl pH 8.2, 1.92 M glycine and 1% (w/v) SDS. Proteins were detected by staining gels with Coomassie Blue in 40% (v/v) methanol and 10% (v/v) acetic acid, and destained in several changes of 7% (v/v) methanol/ 7% (v/v) acetic acid.

2.5.4 Immunoblotting

Immunoblotting was performed as described by Towbin *et al.* (1979). Protein samples were separated by SDS-PAGE and transferred to Immobilon-P nylon membrane (Millipore) using a BioRad Trans-blot apparatus. Electrophoretic transfer was carried out at constant voltage (100V) for 1 h in transfer buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol and 0.02% (w/v) SDS. Following transfer, free protein binding sites were blocked overnight at 4°C in blocking buffer (3% BSA in PBS). The primary antibody (bovine α-FIL) was diluted 1:500 in blocking buffer and incubated with the blot for 2 h at room temperature with gentle

shaking. The blot was washed 5 times (5 min per wash) in PBS. The secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate) was diluted 1:7,500 in blocking buffer and incubated with the blot for 2 h, again at room temperature with gentle shaking. The blot was washed as before, immersed in enzyme substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Gibco BRL) in combination with nitroblue tetrazolium (NBT, Gibco BRL) and allowed to develop in the dark until bands were visible (approximately 5 min). The reaction was terminated with distilled water washes and the filter was air-dried.

2.6 IMMUNOLOGICAL SCREENING

2.6.1 Media

E. coli was grown in LB media containing 1% (w/v) Bacto-tryptone (Oxoid), 0.5% (w/v) Bacto-yeast extract (Oxoid) and 1% (w/v) NaCl, adjusted to pH 7.4 with NaOH. LB solid media was supplemented with 1.5% (w/v) Bacto-agar (Oxoid), while top agar contained 0.7% (w/v) Bacto-agar. SOB contained 2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 10 mM NaCl and 2.5 mM KCl, and was supplemented with 20 mM Mg^{2+} prior to use. A 2 M stock of Mg^{2+} was prepared by combining 1 M $MgSO_4$ and 1 M $MgCl_2$ and sterilised by filtration (Maniatis *et al.*, 1989). SOC, which was used for transformation and preparation of competent cells (2.7.3), is SOB with the addition of 2 mM glucose (Maniatis *et al.*, 1989). A 2 M glucose stock was prepared separately, sterilised by filtration, and added aseptically immediately prior to use. M9 minimal media for growth of *E. coli* JM109 was prepared as described by Maniatis *et al.* (1989). M9 minimal media was supplemented with 1.5% (w/v) Bacto-

agar and after autoclaving, was further supplemented with 0.2% (w/v) glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 μg/ml thiamine.

2.6.2 Preparation of E. coli lysates

100 ml of LB medium was inoculated with strain Y1090 *E. coli* and incubated at 37°C until saturation was achieved. Cells were harvested by centrifugation (5,000*g*, 4°C 10 min) and resuspended in 3 ml TE (50 mM Tris-HCl, pH 8.0, 10 mM EDTA). The cell suspension was freeze-thawed several times and then sonicated (Kontes KT50 cell disrupter) on full power for 6 periods of 20 s at 0°C. The extract was centrifuged (12,000*g*, 4°C, 10 min) and the supernatant was removed and stored at -20°C.

2.6.3 Absorption of antibody with E. coli lysates

The antibody to be used for immunoscreening was diluted 1:10 with blocking buffer (3% BSA in TNT; TNT contained 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20). 25 μl of *E. coli* lysate was added to each ml of antibody solution and incubated at room temperature for 4 h. The absorbed antibody was stored at 4°C, with the addition of 0.05% (w/v) sodium azide, until required.

2.6.4 Preparation of plating bacteria

E. coli strains Y1090 or Y1088 were grown overnight at 37°C in LB medium supplemented with 0.2% (w/v) maltose, 10 mM $MgSO_4$ and 50 μg/ml ampicillin. On the following day, 1 ml of the overnight culture was transferred to 10 ml of fresh LB again supplemented with 0.2% (w/v) maltose, 10 mM $MgSO_4$ and 50 μg/ml ampicillin.

The culture was incubated at 37°C until OD_{550} = 0.5 ± 0.1. Plating bacteria were stored at 4°C and used on the day of preparation.

2.6.5 Library preparation

A random primed goat mammary cDNA library was prepared from lactating goat poly (A) RNA in λgt11. The library contained in excess of 10^6 recombinants with an average insert size of 300 base pairs. The library was provided by Dr M Travers, HRI.

2.6.6 Immunoscreening

Library screening for specific antigen-producing clones was carried out essentially as described by Young & Davis (1983) with some minor modification. Recombinant phage were plated on a lawn of *E. coli* Y1090. Plates were incubated at 42°C for 3 - 4 h to allow expression of fusion proteins. A dry nitrocellulose filter previously saturated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was placed on each plate, and plates and filters were incubated at 37°C for 3 - 8 h. The position of the filter was marked with a needle, removed, and washed twice in TNT for 10 min. Free protein binding sites were blocked by incubation in blocking buffer (3% (w/v) BSA in TNT) for a minimum of 1 h at room temperature with gentle shaking. Following the blocking step the first antibody (bovine α-FIL) was diluted to a final concentration of 1:500 in blocking buffer and incubated, at room temperature, again with gentle agitation, for 2 h. Filters were washed three times (10 min per wash), first in TNT containing 0.1% (w/v) BSA, then in TNT containing 0.1% (w/v) BSA and 0.1% (v/v) Nonidet P40, followed by a final wash in TNT containing 0.1% (w/v) BSA. The second antibody

(anti-rabbit IgG alkaline phosphatase conjugate) was diluted 1:7,500 in blocking buffer and incubated with the filters for 1.5 h at room temperature with gentle agitation. Following incubation with the second antibody, the filters were washed as before. Filters were submerged in enzyme substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Gibco BRL) in combination with nitroblue tetrazolium (NBT, Gibco BRL) and colour development was allowed to proceed in the dark (approximately 5 min). The reaction was terminated by washing the filters in distilled water and air drying. Possible positives were located and removed from the original plates and the recombinants were plaque purified by repeating the same screening procedure until all plaques were positive.

2.6.7 Selection of recombinants of interest

Plaques of interest were picked from agar plates using the wide end of a sterile glass pasteur pipette. The plugs of agar were placed in 1 ml of λ-diluent (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$) containing a few drops of chloroform. Phage were eluted from the plug at 4°C for several hours and cell debris was removed by centrifugation (3,000*g*, room temperature, 10 min).

2.6.8 Preparation of phage lysates

Phage lysates were prepared as described by Davis *et al.* (1986). Bacteriophage of interest were plated at an appropriate dilution in order to obtain individual, well isolated plaques. An agar plug containing a single plaque was placed in a 50 ml plastic tube containing 10 ml of LB supplemented with 10 mM $MgSO_4$. 200 µl of *E. coli*

Y1088 plating cells (prepared as described in section 2.6.2) were added and the cultures were incubated at 37°C, with vigorous shaking for 6 -12 h, or until visible lysis occurred. After lysis, 100 µl chloroform was added and cultures were incubated at 37°C with shaking for 2 min. Bacterial debris was removed by centrifugation (3,000*g*, room temperature, 10 min) and the supernatant was removed to a fresh tube. The lysates were supplemented with 10 mM $MgSO_4$, titred by serial dilution and plating, and stored at 4°C.

2.6.9 Isolation of DNA from phage lysates

DNA was prepared from phage lysates as described by Davis *et al.* (1986). 320 µl of fresh DNase I (1mg/ml in λ diluent) and 10 ml of λ diluent were added to each 10 ml of lysate, and incubated at room temperature for 15 min. Phage were precipitated by addition of 2 ml of 5 M NaCl and 2.2 g of solid polyethylene glycol (PEG) 6000 and incubation on ice for 15 min. The phage pellet was recovered by centrifugation (12,000*g*, 4°C, 10 min), resuspended in 300 µl of λ diluent and transferred to a microcentrifuge tube. Protein was removed from the preparation by extraction with an equal volume of chloroform. 15 µl of 0.5 M EDTA pH 8.0 and 30 µl of 5 M NaCl were added to the aqueous phase and the mixture was extracted with 350 µl of Tris-saturated phenol. The phases were separated by centrifugation (12,000*g*, room temperature, 5 min) and the upper aqueous phase was removed to a fresh microtube. DNA was precipitated on wet ice by the addition of 875 µl of ethanol. DNA was

recovered by centrifugation (12,000g, 4°C, 5 min) rinsed with 150 µl of 80% (v/v) ethanol, dried under vacuum and resuspended in 50 µl of water.

2.6.10 Restriction enzyme digestion

Restriction endonuclease digestion of DNA was carried out according to the manufacturer's instructions. A typical digestion mixture contained 0.1 - 5 µg of DNA, 10 µl of the appropriate 10x buffer (supplied with the restriction enzyme), 10 µl of 1 mg/ml BSA and 0.2 - 10 units of restriction endonuclease in a total volume of 100 µl. Digests were incubated at 37°C for 1 - 12 h, depending on the amount of enzyme added.

2.6.11 Electrophoresis of DNA

DNA was separated by electrophoresis through agarose gels containing 0.7% - 1.0% (w/v) agarose in TBE buffer (50 mM Tris-HCl, pH 7.5, 50 mM Boric acid, 10 mM EDTA) with 0.5 µg/ml ethidium bromide. Electrophoresis was carried out for 2 h in 1 x TBE at approximately 70 volts. Electrophoresed DNA was visualised on a short-wave UV transilluminator and photographed using a Polaroid land camera and Polaroid type 665 film.

2.7 PREPARATION FOR DNA SEQUENCING

2.7.1 Polymerase chain reaction

Amplification reactions were performed using the primers BG1 and BG2 and template DNA prepared from phage lysates. The upstream primer, BG1 (5'

ATATGGGGATTGGTGGCGACGACTCCTGGA 3') and downstream primer, BG2 (5' GACACCAGACCAACTGGTAATGGTAGCGAC 3') were complementary to nucleotide sequences 2969-2998 and 3035-3064 respectively of the β-galactosidase gene of λgt11. The amplification mixture contained 1 ng of template DNA, 0.2 μM each primer, 10 μl of 10x buffer (supplied with the enzyme), 2.5 mM $MgCl_2$ and 2 mM of each deoxynucleoside triphosphate, in a total volume of 100 μl. The mixture was denatured at 95°C for 5 min, and after cooling to 72°C, 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase were added. The reaction mix was overlaid with 100 μl of light mineral oil and amplified using a Crocodile II thermocycler (Appligene, Durham, UK). The thermocycler was programmed for 45 cycles of 30 s at 93°C, 30 s at 55°C and 30 s at 72°C, using the fastest possible transition times, followed by a final 10 min at 72°C. PCR products were analysed by electrophoresis in 1% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide.

2.7.2 Ligation of amplified DNA into pGEM 5zf

DNA amplified using the polymerase chain reaction was ligated into the pGEM 5zf (+) series of phagemid vectors using T4 DNA ligase (Kovalic *et al.*, 1991). The ligation mixture, 100 ng of vector, 125 ng of insert DNA, 0.5 μl of 10x ligation buffer, 1 mM hexamine cobalt chloride, 0.5 mM ATP, 30 mM KCl and 5 units of T4 DNA ligase, in a total volume of 5 μl, was incubated at 14°C for 12 - 24 h. 10x ligation buffer contained 250 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$ and 5 mM DTT. One μl of the reaction mixture was used for electroporation of *E. coli* JM109 cells (section 2.7.3).

2.7.3 Transformation and preparation of competent cells

Cells were transformed and competent cells prepared as described by Hanahan (1988). Frozen JM109 cells were streaked on M9 minimal media plates and grown for 36 h at 37°C. 50 ml SOB was inoculated with 2-3 JM109 colonies. This culture was incubated at 37°C, with shaking, for approximately 2 h until $OD_{550} = 0.4 \pm 0.1$. The culture was transferred to a 50 ml plastic tube and chilled on ice for 10 - 15 min. Cells were pelleted by centrifugation (3,000*g*, 4°C, 15 min), and resuspended in 1/3 volume (16.6 ml) of TFB. TFB contains 100 mM KCl, 45 mM $MnC1.4H_20$, 10 mM $CaC1_2.2H_2O$, 3 mM $HACoC1_3$ and 10 mM K-MES. Following the addition of TFB, the cell suspension was incubated on ice for 15 min, cells were collected as before and resuspended in 1/12.5 volume (4 ml) of TFB. 140 µl DnD (1 mM DTT, 90% (v/v) DMSO, 10 mM potassium acetate, pH 7.5) was added and incubated on ice for 10 min. A further aliquot (140 µl) of DnD was added and incubated on ice for 20 min. 200 µl of the cell suspension was transferred to a 15 ml plastic tube, and 1 µl of ligation mix was added and incubated on ice for 30 min. Cells were subjected to heat shock by placing in a 42°C water bath for 90 s. The heat shock was quenched by incubation on ice for a minimum of 2 min. 800 µl of SOC was added to the heat-shocked cells and the cells incubated at 37°C for 30-60 min. 200 µl of this cell suspension was added to 2.5 ml of top agar containing 50 µg/ml ampicillin, 350 µg/ml X-gal and 140 µg/ml IPTG. Plates were incubated overnight at 37°C. Transformed cells grew as white plaques, those that were not transformed grew as blue plaques.

2.7.4 Preparation of plasmid DNA

Plasmid DNA was isolated from transformed cells using a modification of the method of Birnboim & Doly (1979). Colonies of interest were grown overnight in 5 ml of LB containing 50 μg/ml ampicillin. 5 μl of this bacterial suspension was used to inoculate 50 ml of LB containing 50 μg/ml ampicillin, and incubated overnight at 37°C. Next day, 40 ml of the culture was transferred to a sterile SS34 tube and cells were pelleted by centrifugation (8,000*g*, 4°C, 5 min). The cell pellet was resuspended in 1 ml of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 5 mg/ml lysozyme and incubated at room temperature for 5 min. 2 ml of freshly prepared solution II (1% (w/v) SDS, 0.2 M NaOH) was added and incubated on ice for 10 min. After addition of 1.5 ml of cold solution III (3 M potassium acetate:2 M acetic acid, pH 4.8 - 5.0) the cell suspension was again incubated on ice for 10 min. Cell debris was collected by centrifugation (10,000*g* 4°C, 30 min) and the supernatant transferred to a sterile Corex tube. DNA was precipitated at -70°C with the addition of 2.5 volumes of absolute alcohol. The DNA was recovered by centrifugation (12,000*g*, 4°C, 30 min), dried under vacuum, resuspended in 400 μl water and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v). The aqueous layer, containing DNA, was further extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and precipitated at -70°C with the addition of 0.1 volume of 3 M sodium acetate, pH 6.0 and 2 volumes of absolute alcohol. The DNA was recovered as before. Contaminating RNA was removed by resuspending the DNA in 100 μl water containing 20 μg/ml RNase A and incubating at 37°C for a minimum of 1 h. DNA was visualised by electrophoresis on a 1% (w/v) agarose gel in TBE buffer.

2.7.5 Quantification of plasmid DNA

Plasmid DNA was quantified by running a known volume of plasmid DNA on a 1% (w/v) agarose gel and comparing the ultraviolet fluorescence produced with that from 1 μg of *Hin*d III/*ECo*R I digested λ DNA run on the same gel.

2.7.6 Alkali denaturation of supercoiled plasmid DNA

To prime efficiently double stranded plasmids must be converted to a single-stranded form before sequencing is attempted. This is accomplished by alkali denaturation of the supercoiled plasmid DNA. 4 μg (supercoiled) plasmid DNA was placed in a total volume of 18 μl water. 2 μl of 2 M NaOH, 2 mM EDTA solution was added and incubated at room temperature for 5 min. The reaction mix was neutralised by adding 2 μl of 2 M ammonium acetate, pH 4.6. The DNA was precipitated at -70°C by the addition of 75μl of absolute alcohol. DNA was recovered by centrifugation (12,000*g*, 4°C, 10 min), washed with 200 μl of cold 70% (v/v) ethanol, dried under vacuum and resuspended in 17.5 μl of water, ready for sequencing.

2.7.7 DNA sequencing

DNA was sequenced using the chain termination method of Sanger *et al.* (1977) with the aid of sequencing grade *Taq* DNA polymerase (Innis *et al.*, 1988). 4 μg of plasmid DNA was denatured as described in section 2.7.6 and annealed to either a reverse or forward pUC/M13 sequencing primer (Promega). The annealing reaction mix, 17.5 μl (4 μg) of alkali denatured plasmid DNA, 5 μl of 5x Taq buffer, 2 μl of extension mix

and 0.5 µl of 10 µg/ml pUC/M13 primer (reverse or forward), was incubated at 37°C for 10 min. 5x Taq buffer was supplied with the enzyme and contained 250 mM Tris-HCl, pH 9.0 and 50 mM $MgCl_2$. The extension mix contained 7.5 µM each of dGTP, dTTP and dCTP. Following the annealing step, 5 µCi [α-^{35}S]ATP was added to the annealed primer and template. Five units of sequencing grade *Taq* were added and the reaction mix was incubated at 37°C for 5 min. Following extension/labelling, the reaction mix was divided into four aliquots and each aliquot was placed in a microtube containing 1 µl of the appropriate nucleotide mix (G, A, T or C) as described in table 2.1. This termination reaction was incubated at 70°C for 15 min, and 4 µl stop solution (10 mM NaOH, 95% (v/v) formamide, 0.05% (w/v) Bromophenol blue and 0.05% (w/v) xylene cyanol) was added. Each sequencing reaction (G, A, T and C) was incubated at 80°C immediately prior to loading on the sequencing gel.

6% (w/v) polyacrylamide sequencing gels were prepared in TBE buffer (50 mM Tris-HCl, pH 7.5, 50 mM Boric acid, 10 mM EDTA) containing 48% (w/v) urea. Gels were polymerised by addition of 0.05% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. Electrophoresis was carried out at constant power (50 watts) for 6 h in 1x TBE buffer. The gels were fixed for 30 min in 10% (v/v) methanol and 10% (v/v) acetic acid in water, mounted on 3 MM paper (Whatman) and dried using a BioRad gel drier. Autoradiography was performed as described previously (section 2.2.7).

Component	G Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	C Nucleotide Mix
ddGTP	25 μM	-	-	-
ddATP	-	350 μM	-	-
ddTTP	-	-	300 μM	-
ddCTP	-	-	-	160 μM
7-deaza dGTP	25 μM	250 μM	250 μM	250 μM
dATP	250 μM	25 μM	250 μM	250 μM
dTTP	250 μM	250 μM	25 μM	250 μM
dCTP	250 μM	250 μM	250 μM	25 μM

Table 2.1 Nucleotide mix formulation for DNA sequencing.

2.7.8 DNA analysis

Sequence data was compared with known sequences by scanning the EMBL database with the Pearson FASTA programme using the SEQNET facility on the Daresbury database.

2.7.9 163 base pair probe

Plasmid DNA, prepared as described in section 2.7.4, was digested with the restriction enzyme *Nco* I and electrophoresed on a low melting point agarose (1%, v/v) gel in TAE buffer. The band corresponding to the inserted DNA was excised and labelled with [α-^{32}P]dCTP as described in section 2.2.5. This cDNA probe was used to identify similar recombinants isolated by immunoscreening (section 2.6.4).

2.7.10 Screening a cDNA library with a radiolabelled cDNA probe

Recombinant phage were plated on a lawn of *E. coli* Y1090. Plates were incubated for 12 - 16 h at 37°C. A dry nitrocellulose filter was placed on each plate and its position marked with a needle. The filter was removed after 30 s contact with the plate. The colonies were lysed by sequential incubations on Whatman 3 MM paper saturated with first 0.5 N NaOH and 1.5 M NaCl for 5 min, followed by 0.5 M Tris-HCl, pH 8.0 and 1.5 M NaCl for 10 min, and then 2x SSC for 5 min. Between each incubation the filters were blotted on dry 3 MM paper. The DNA was bound to the nitrocellulose membrane using a UV crosslinker (Spectrolinker) according to the manufacturer's instructions. Labelling of cDNA probes, hybridisation and autoradiography were carried out as described in sections 2.2.5, 2.2.6 and 2.2.7 respectively.

2.8 LIBRARY CONSTRUCTION

2.8.1 Preparation of poly (A) RNA

Poly (A) RNA was prepared from total goat mammary RNA using a Promega Poly Attract system IV mRNA isolation kit, according to the manufacturer's instructions.

2.8.2 First strand cDNA synthesis

First strand cDNA was synthesised using MMLV reverse transcriptase and oligo dT_{12-18} using a modification of the method of Krug & Berger (1987). A known quantity of poly (A) RNA (maximum 2 µg) was placed in a sterile microtube and denatured at 70°C for 2 min. 4 µl of 5x reverse transcriptase buffer, 0.1 M DTT and 5 µl of 100 µg/ml OdT_{12-18} primer were added to the denatured RNA, and the primer was annealed to the RNA at 42°C for 10 min. After the annealing step 1 µl of 10x dNTPs (10 mM each dNTP), 10 units of RNAsin, 5-10 µCi [^{32}P]dCTP and 400 units of MMLV reverse transcriptase (Gibco BRL) were added in a total volume of 20 µl, and cDNA synthesis allowed to progress at 42°C for 1.5 h. 5x reverse transcriptase buffer was provided with the enzyme and contained 250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$.

2.8.3 Second strand cDNA synthesis

Double stranded cDNA was synthesised using RNase H and DNA polymerase I as described by Gubler (1987). The reaction mix contained 19 µl of first strand cDNA, 47.5 µl of 2x second strand buffer, 0.95 µl of 4 mM dNTPs, 0.95 µl of RNase H, 23

units of DNA polymerase I, 5 units of DNA ligase and 10-15 μCi [^{32}P]dCTP in a total volume of 95 μl, and was incubated for 1 h at 12°C, followed by 1 h at 22°C. 2x second strand buffer contained 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 200 mM KCl and 0.1 mg/ml BSA. The ends of the double stranded cDNA were made flush by the addition of 20 units T4 polymerase, and incubation of the cDNA at 22°C for a further 30 min. 1 μl of the synthesis reaction was removed to assess the efficiency of cDNA synthesis. The double stranded cDNA was precipitated with the addition of 2 volumes of absolute alcohol and recovered by centrifugation (12,000*g*, 4°C, 30 min). The cDNA was washed with 100 μl of 80% (v/v) ethanol, dried under vacuum and resuspended in 100 μl of water.

2.8.4 Efficiency of first and second strand synthesis

1 μl from each of the first and second strand synthesis reactions were reserved to check both the percentage of first and second strand cDNA synthesised and the size of the cDNA. Unincorporated nucleotides were removed by gel filtration through a 1 ml column of Sephadex G50 in NE (50 mM NaCl, 1mM EDTA, pH 7.0), and the amount of radioactivity incorporated into newly synthesised cDNA was determined by Cerenkov counting. The size of cDNA synthesised in the first and second strand reactions was assessed by electrophoresis on an alkaline agarose gel (1% w/v) in alkaline buffer (60 mM NaOH, 2 mM EDTA). The cDNA was loaded on an equal cpm basis and resolved in 1x alkaline agarose buffer at approximately 50 volts for 2 h. Hind III/ECoRI digested λ DNA markers were labelled with [α^{32}P]dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Cobianchi & Wilson, 1987), and

included in the gel in order to assess the size of the synthesised cDNA. Gels were fixed for 30 min in 10% (v/v) methanol and 10% (v/v) acetic acid in water, dried and exposed to X-ray film as described in section 2.2.7.

2.8.5 Purification of the double stranded cDNA

Double stranded cDNA was extracted with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1 v/v), and the upper aqueous layer was extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). The organic phase was back-extracted with 100 µl TE, pH 8.0 and the upper aqueous layer extracted with an equal volume of chloroform:isoamyl alcohol. The aqueous layers were pooled and the cDNA was precipitated by the addition of 2 volumes of absolute alcohol. The cDNA was recovered by centrifugation, dried under vacuum and resuspended in 100 µl of ligation buffer. Ligation buffer contained 66 mM Tris-HCl, pH 7.6, 1 mM spermidine, 10 mM $MgCl_2$, 15 mM DTT and 200 µg/ml BSA. The cDNA was purified by centrifugation (400*g*, room temperature, 2 min) through a S300 spun column (Pharmacia) equilibrated with ligation buffer.

*2.8.6 Addition of ECo*R I/*Not* I *adaptors*

To prepare blunt ended cDNA for insertion into the *ECo*R I site of λgt11, an *ECo*R I/*Not* I adaptor was ligated to each end of the cDNA molecule using T4 DNA ligase. The blunt-ended cDNA was purified on a spun column as described in section 2.8.5. The ligation mix, 100 µl of column effluent, 2.5 µl of *ECo*R I/*Not* I adaptors, 1 µl of 10 mM ATP and 3 µl of T4 DNA ligase, was incubated for 16 h at 12°C. Following

overnight incubation the T4 DNA ligase was denatured by heating at 65°C for 30 min and the *ECo*R I-ended cDNAs were phosphorylated using T4 polynucleotide kinase. 10 µl of 10 mM ATP and 1 µl of T4 polynucleotide kinase were added directly to the cDNA and incubated at 37°C for 30 min. The T4 kinase was then inactivated by heating to 65°C for 35 min. The cDNA was extracted with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1 v/v/v). Unligated adaptors were removed from the aqueous layer by centrifugation (400*g*, room temperature, 2 min), through a S300 spun column equilibrated with STE buffer. STE buffer contained 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl. The column effluent was precipitated by the addition of 2 volumes of absolute alcohol at -70°C, recovered by centrifugation (12,000*g*, 4°C, 30 min), rinsed with 100 µl of 80% (v/v) alcohol, dried under vacuum and resuspended in water at a concentration of 10 ng/µl, ready for insertion into a λgt11 vector.

2.8.7 Insertion of cDNA into a λgt11 vector

The cDNA was prepared as described above (section 2.8.4), and inserted into a λgt11 vector using T4 DNA ligase. Small test reactions were set up to identify the optimal conditions for ligation of the cDNA and vector. The ligation reaction contained 0.4 µl (200 ng) of λgt11 vector, varying amounts (2 - 6 ng) of double stranded cDNA, 0.2 µl of 10x ligation buffer and 0.2 µl of T4 DNA ligase in a total volume of 2 µl. The ligation mixture was incubated for 16 h at 12°C. 10x ligation buffer contained 400 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP and 0.5 mg/ml BSA. Following incubation, 1 µl of each ligation mix was placed in a fresh tube, 6 µl of

Packagene extract (Promega) was added and the reactants were incubated at 22°C for 2 h. 100 µl of λ diluent and 20 µl of chloroform were added to the packaging extract and titration of the packaged phage were carried out on LB plates. Appropriate dilutions of packaged phage were made (1:1,000 or 1:10,000) in λ diluent. 100 µl of diluted phage was added to 200 µl of *E. coli* Y1088 plating bacteria (prepared as described in section 2.6.2) and plated in 2.5 ml of top agar containing 50 µg/ml ampicillin, 140 µg/ml IPTG and 350 µg/ml X-gal. Plates were incubated overnight at 37°C and scored for the number of clear plaques (non-recombinant phage) versus the number of blue plaques (recombinant phage).

Once optimal conditions had been determined a larger scale ligation reaction was set up. The ligation reaction contained 2 µl (1 µg) of λgt11 vector, 20 ng of double stranded cDNA, 1 µl of 10x ligation buffer and 1 µl of T4 DNA ligase in a total volume of 10 µl. The ligation mix was incubated at 12°C for 36 h and then packaged using Gigapack packaging extracts (Stratagene, Cambridge, UK) and incubated at 22°C for 2 h, according to the manufacturer's instructions. 500 µl of λ diluent and 20 µl of chloroform were added to the packaging mix and the phage were titred as before.

2.8.8 Analysis of inserted DNA

The average insert size of the cDNA library was determined by restriction enzyme digestion of the phage DNA. Phage lysates were prepared from recombinant phage (section 2.6.6), DNA was isolated from these phage lysates (section 2.6.7) and the

inserted DNA was excised from the λgt11 vector using the restriction enzyme *Not* I. Analysis of the inserted cDNA is discussed in detail in Chapter six.

2.8.9 Amplification of a cDNA library

Y1088 plating bacteria were prepared as described in section 2.6.2. 10^5 phage were placed in a sterile tube containing 600 µl of E. *Coli* Y1088 plating bacteria and incubated at 37°C for 5 min. 6.5 ml of top agar was added to each tube and the contents poured onto a 150 mm agar plate. Plates were incubated at 37°C for approximately 6 h until the plaques were approximately 0.5 mm in diameter. The plates were overlaid with 12 ml of λ diluent and stored at 4°C overnight. Next day, the buffer was collected, the plates rinsed with 4 ml λ diluent and the buffer pooled, and chloroform was added to a final concentration of 5% (v/v). The library was incubated at room temperature for 15 min and bacterial debris was removed by centrifugation (3,000*g*, room temperature, 10 min). The upper layer was transferred to a fresh tube and chloroform added to a final concentration of 0.5% (v/v). The amplified library was stored at 4°C.

2.9 OLIGONUCLEOTIDE SCREENING

2.9.1 Oligonucleotide synthesis

Sense and antisense oligonucleotides were purchased from Pharmacia. Oligonucleotides were constructed on the basis of known protein sequence. Oligonucleotide sequences are discussed in detail in Chapter six.

2.9.2 5' end-labelling of synthetic oligonucleotides

Oligonucleotide probes were labelled to high specific activity (typically 1 x 10^6 counts per ml of hybridisation buffer) with [γ-^{32}P]ATP using T4 polynucleotide kinase, as described by Cobianchi & Wilson (1987). The kinase reaction mixture, 125 ng of oligonucleotide, 1.25 µl of 10x buffer, 37.5 µCi [γ-^{32}P]ATP at 3000 Ci/mmol and 5 units of T4 polynucleotide kinase, in a final volume of 12.5 µl, was incubated at 37°C for 30 min. 10x kinase buffer contained 500 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine hydrochloride and 1 mM EDTA. Unincorporated nucleotides were removed as described in section 2.2.5. Ethanol precipitation of the labelled oligonucleotide was carried out prior to hybridisation.

2.9.3 Hybridisation conditions

For hybridisation with oligonucleotide probes, blots were incubated in aqueous pre-hybridisation buffer (6x SSC, 5x Denhardt's, 0.1% (w/v) SDS, 200 µg/ml denatured herring sperm DNA and 0.05% (w/v) pyrophosphate tetrasodium salt), for a minimum of 4 h. The precipitated probe was recovered by centrifugation (12,000*g*, 4°C, 30 min) and resuspended in 50 µl of water. Hybridisations were carried out in hybridisation buffer (same composition as pre-hybridisation buffer) for 16-24 h at an appropriate temperature determined by the nucleotide composition of the probe. Hybridisations were performed at 5°C below the minimum value for the thermal melting point of the oligonucleotide, T_m, as determined using a Genequant DNA calculator (Pharmacia) according to the manufacturer's instructions. Membranes were washed in 2x SSC/0.1% (w/v) SDS three times at room temperature, followed by a final wash in

0.2x SSC/0.1% (w/v) SDS, at a temperature equal to or greater than the T_m as appropriate. Autoradiography was carried out as described previously (section 2.2.7).

2.9.4 Amplification of cDNA 3' ends (3' RACE)

First strand cDNA was prepared as described by Frohman (1990). 1 µg of poly (A) RNA was denatured at 65°C for 3 min. The denatured RNA was then added to a mixture containing 2 µl of 10x reverse transcriptase buffer, 10 units of Rnasin, 0.5 µg of dT_{17}-adapter primer and 10 units of MMLV reverse transcriptase in a final volume of 20 µl. 10 x reverse transcriptase buffer contained 500 mM Tris-HCl, pH 8.15, 60 mM $MgCl_2$, 400 mM KCl, 1 mM DTT and 10 mM each dNTP. The cDNA synthesis reaction mix was incubated at 42°C for 1 h, and then at 52°C for 30 min. The cDNA pool was diluted to 1 ml with TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) and stored at 4°C until required. Amplification of cDNA 3' ends was carried out as described by Frohman (1990). The amplification mixture contained 5 µl of 10x PCR buffer, 5 µl of DMSO, 5 µl of 10x dNTPs (15 mM each dNTP), 30 µl of water, 1 µl of adapter primer (25 pmol/µl), 1 µl of gene specific primer (25 pmol/µl) and 1-5 µl of cDNA pool. 10x PCR buffer contained 670 mM Tris-HCl, pH 8.8, 67 mM $MgCl_2$, 1.7 mg/ml BSA and 166 mM $(NH_4)_2SO_4$. The mixture was denatured at 95°C for 5 min and, after cooling to 72°C, 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase were added. The amplification mix was overlaid with 100 µl of light mineral oil and subjected to 40 cycles of 40 s at 95°C, 60 s at 55°C, and 3 min at 72°C, using the fastest possible transition times and followed by a final 10 min at 72°C. PCR products were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and

analysed by electrophoresis in 1% (w/v) agarose gels containing ethidium bromide. The nucleotide sequences of the dT_{17}-adapter primer and the adapter primer are as described by Frohman (1990), while the sequences of the gene-specific primers are discussed in detail in Chapter six.

Chapter Three

Effect of milking strategies on mammary development in lactating goats

3.1 INTRODUCTION

The rate of milk secretion in dairy animals is regulated by mechanisms sensitive to the frequency and completeness of milk removal (Wilde & Peaker, 1990; Wilde *et al.*, 1990). For example, milking one gland of lactating goats thrice instead of twice daily stimulated milk secretion unilaterally within hours (Blatchford & Peaker, 1982; Linzell & Peaker, 1971). This local increase in milk yield is maintained for as long as frequent milking is applied, and is sustained by developmental responses in the frequently milked gland. Ten days of frequent milking elicited a significant increase in secretory cell differentiation, as measured by the activities of several key enzymes involved in milk synthesis (Wilde *et al.* 1987), and prolonged frequent milking was accompanied by an increase in the number of secretory cells in the more frequently milked gland (Knight *et al.* 1990; Wilde *et al.*, 1987). In addition, after 22 weeks of more frequent milking, higher activities of two key enzymes, fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), were associated with an increase in abundance of messenger RNA (mRNA) for these proteins, indicating that the effects of milking frequency are exerted at the level of gene expression (Travers & Barber, 1993; Wilde *et al.*, 1990). We wished to determine if frequency or completeness of milk removal also regulates the expression of milk protein genes and if so, whether this effect is also mediated by FIL, the Feedback Inhibitor of Lactation. To address the first objective lactating goats were subjected to frequent milking, infrequent milking, suckling or combinations thereof. Northern analysis was used to measure the abundance of mRNAs encoding α_{s1}-casein

(a major milk protein) and α-lactalbumin, a component of lactose synthase and therefore a key determinant of milk volume (see section 1.2.1).

3.2 EXPERIMENTAL DESIGN

3.2.1 Manipulation of milking frequency

Two experiments were performed. In a preliminary study, different milking frequencies were applied unilaterally for 9 days in two groups (n = 4, each group) of goats in early lactation (Bryson *et al.*, 1993). One group was milked once and twice daily in individual glands, the other group twice and thrice. The aim of this study was to determine whether the acute effect on milk secretion was independent of or dependent on changes in mammary gene expression.

In a second longer-term experiment, three different milking strategies were compared with once daily milking over a period of six weeks. The aim of this study was to determine whether longer term changes in milking frequency are accompanied by alterations in milk protein gene expression. After parturition, goats were milked twice daily at 0800 and 1600 h for a maximum of nine days. This pre-treatment period was sufficient to establish a reliable pre-treatment milk yield before switching one gland to once daily milking and the contralateral gland to one of the three experimental strategies. Following the pre-treatment period, the left gland was milked once daily (1600 h) and the right gland milked thrice daily (n = 4), drained continuously (n = 3) or suckled by kids (n = 3). In the suckled and drained groups the treatment gland was also hand milked once daily to ensure complete emptying of the gland once in each 24

h period. This allowed estimation of the efficiency of suckling by kids or the extent of drainage via the cannula. The treatment regime was carried out on six days out of seven.

Day seven, termed the "measurement" day, was used to determine the milk yield in the treated gland. On this day the left gland was milked once daily at 1600 h as usual. All treatment glands were milked thrice daily at 1600, 0000 & 0800 h with exogenous oxytocin (0.4 IU, Intervet, Cambridge, UK) to ensure complete milk removal. During this 24 h period kids were separated from mothers and teat cannulas, where appropriate, removed or plugged.

3.2.2 Effects on milk yield

The unilateral effect of changes in milking frequency was determined as a relative milk yield quotient (RMYQ) as described by Linzell & Peaker (1971):

$$\mathbf{RMYQ}=(a_2.b_1)(a_1.b_2)^{-1}$$

where a_1 and a_2 are the yields of the right gland before and after treatment, and b_1 and b_2 the yield of the left gland in the same two periods. Pre-treatment yields were calculated as the average milk yield for the three days prior to treatment, and post treatment yields as the average milk yield for the final three days of treatment. An RMYQ value >1 indicates an increase in milk yield of the right (test) gland relative to that of the left (control) gland. Conversely, an RMYQ value <1 indicates a decrease in yield of the test gland relative to that of the control gland.

3.2.3 Effects on milk protein gene expression

The effect of milking frequency on milk protein gene expression was determined by northern blot analysis. Total cellular RNA was isolated from biopsy tissue taken from goat mammary glands milked differentially for 9 days or 6 weeks. Alpha-lactalbumin and α_{s1}-casein mRNA abundance was expressed as the integrated area of the 0.7 kb α-lactalbumin mRNA and 1.2 kb α_{s1}-casein mRNA, normalised against 28S ribosomal RNA, taking into consideration the DNA content of the tissue and the yield of RNA. Milk protein gene expression was expressed as units of mRNA abundance per mg DNA.

3.3 RESULTS

3.3.1 Short-term study

Pre-treatment milk yields were established during a period (3 days) of bilateral twice daily milking at a time of increasing milk yield. Thereafter, milking one gland once daily prevented the further increase in milk yield seen in the contralateral twice daily milked gland, such that animals milked once and twice daily showed a positive RMYQ of 1.42 ± 0.21 (mean ± SEM, n = 4). While milk yield increased in the twice daily milked gland, there was no significant increase in the abundance of either α-lactalbumin or α_{s1}-casein mRNA. Goat B9 (figure 3.1) displayed the largest differential in milk yield between the two glands (RMYQ = 1.83) over the treatment period, yet no increase in milk protein gene expression was observed.

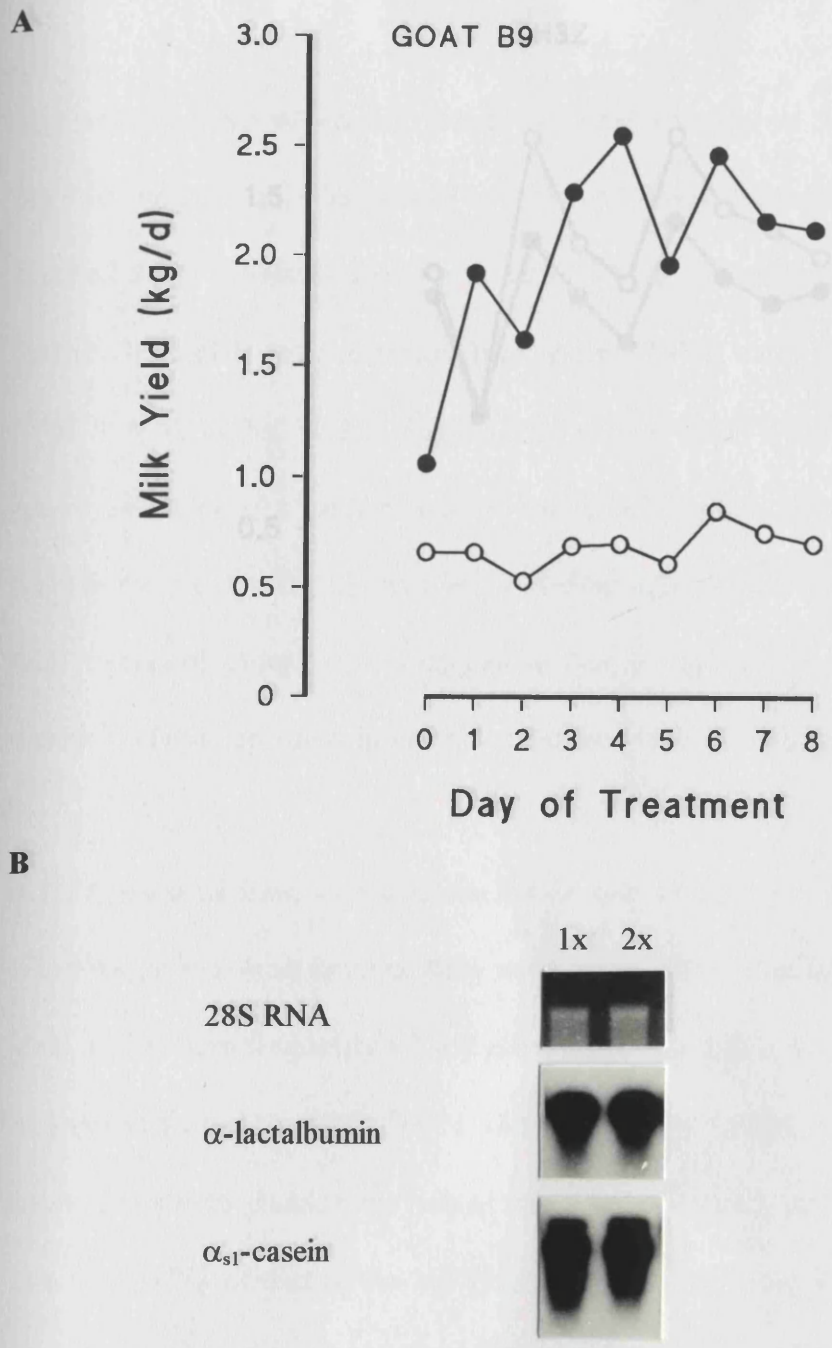
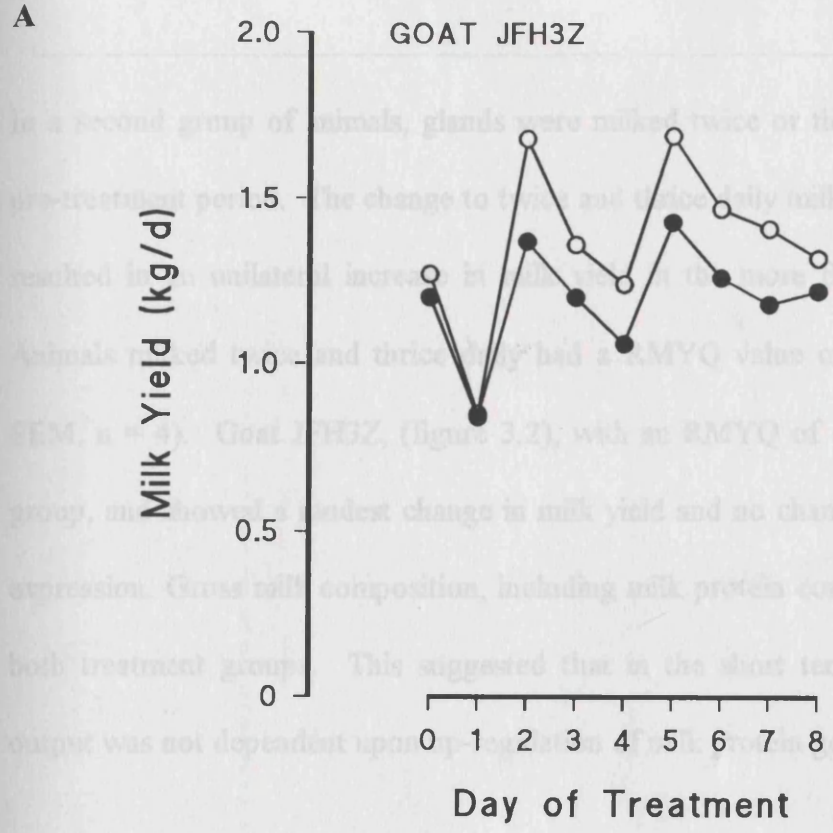


Figure 3.1 Effect of short-term unilateral once and twice daily milking on milk yield (A) and milk protein gene expression (B) in one lactating goat. The right gland was milked twice (2x) daily (-●-) while the left gland was milked once (1x) daily (-o-) for 9 days.



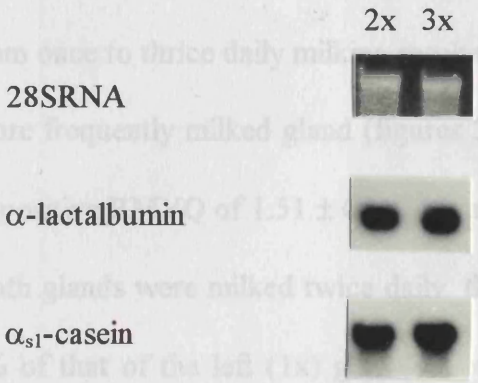


Figure 3.2 Effect of short-term unilateral twice and thrice daily milking on milk yield (A) and milk protein gene expression (B) in one lactating goat. The right gland was milked thrice (3x) daily (-o-) while the left gland was milked twice (2x) daily (-●-) for nine days.

In a second group of animals, glands were milked twice or thrice daily following the pre-treatment period. The change to twice and thrice daily milking of individual glands resulted in an unilateral increase in milk yield in the more frequently milked gland. Animals milked twice and thrice daily had a RMYQ value of 1.14 ± 0.03 (mean ± SEM, n = 4). Goat JFH3Z, (figure 3.2), with an RMYQ of 1.12 was typical of this group, and showed a modest change in milk yield and no change in milk protein gene expression. Gross milk composition, including milk protein content, was unaffected in both treatment groups. This suggested that in the short term the increase in milk output was not dependent upon up-regulation of milk protein gene expression.

3.3.2 Long term study - once versus thrice daily milking

The change from once to thrice daily milking resulted in a unilateral increase in the milk yield of the more frequently milked gland (figures 3.3 & 3.4). Animals treated in this way showed a positive RMYQ of 1.51 ± 0.24 (mean ± SEM, n = 4). At the start of the study, when both glands were milked twice daily, the yield of the right (3x) gland was 104.7 ± 10.7% of that of the left (1x) gland. However by the end of the treatment period the yield of the thrice daily milked gland had increased to 158 ± 24.4% of that of the once daily milked gland. Individual RMYQ values and pre- and post-treatment milk yields are shown in table 3.1.

Northern blot analysis of total RNA isolated from each gland before and after treatment (figure 3.5) indicated that six weeks of differential milking produced an increase in the abundance of α-lactalbumin mRNA/mg DNA in the thrice daily milked

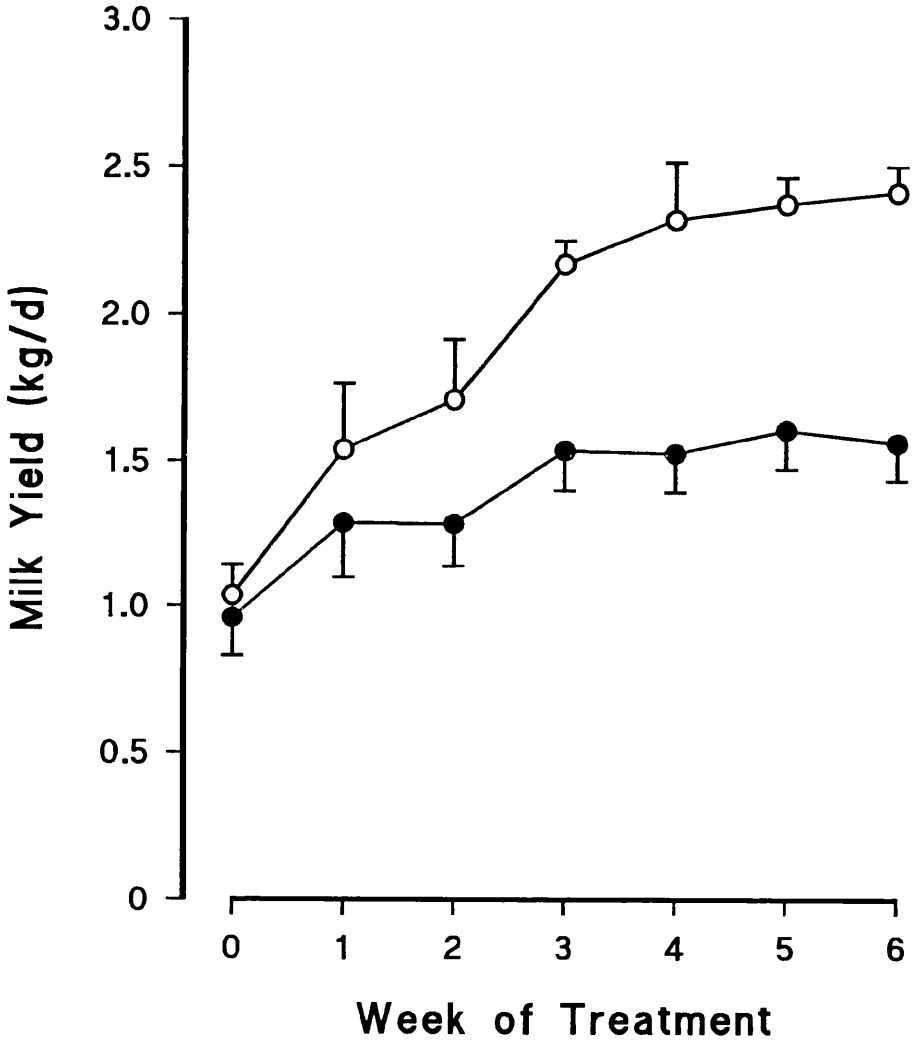
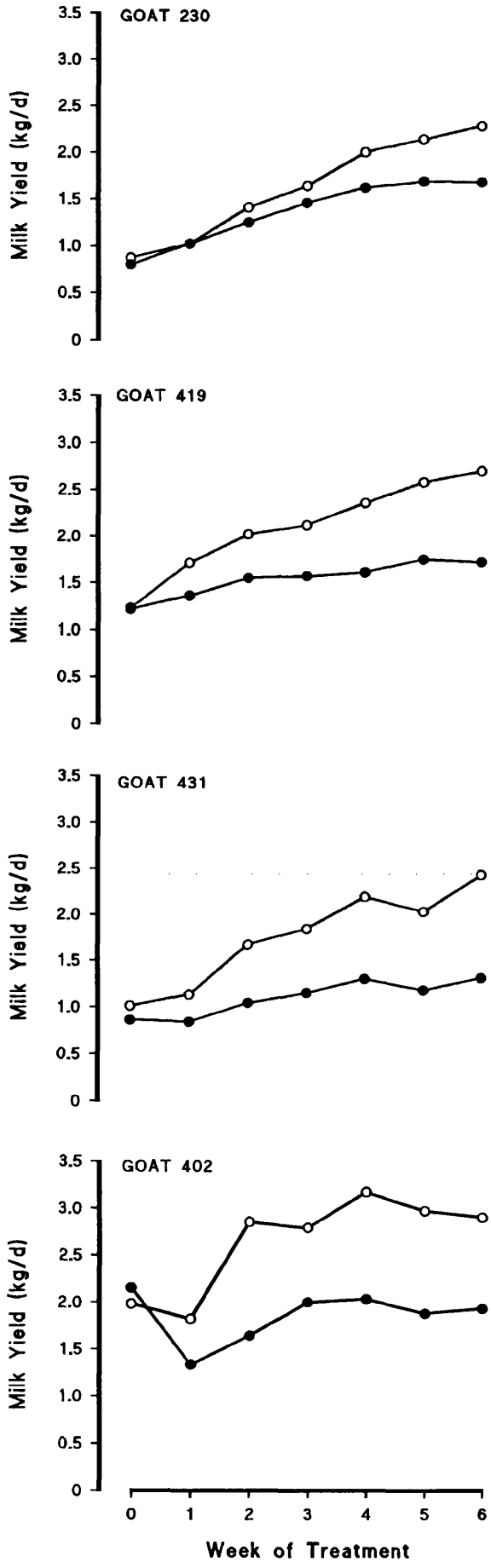


Figure 3.3 Effect of unilateral once and thrice daily milking on milk yield. The right gland was milked thrice daily (-o-), while the left gland was milked once daily (-●-) for 6 weeks. Values are the mean ± SEM for 4 animals.

Figure 3.4 Effect of milking frequency on individual gland milk yield in four lactating goats. The right gland was milked thrice daily (-o-), while the left gland was milked once daily (-●-) for 6 weeks.


GOAT 230
GOAT 419
GOAT 431
GOAT 402
Milk Yield (kg/d)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 1 2 3 4 5 6
Week of Treatment

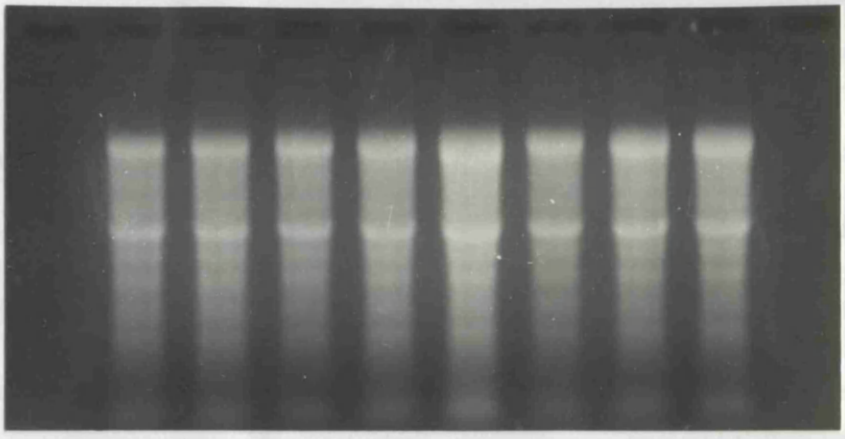
Goat	Gland	Treatment	Milk Yield pre (kg/d)	Milk Yield (post (kg/d)	RMYQ
230	R	3x	0.87	2.24	1.19
	L	1x	0.80	1.73	
419	R	3x	1.23	2.53	1.52
	L	1x	1.22	1.65	
431	R	3x	1.01	2.47	1.60
	L	1x	0.86	1.31	
402	R	3x	1.98	3.05	1.76
	L	1x	2.15	1.88	

Table 3.1 Effect of different milking frequencies, applied unilaterally, on individual gland milk yield in four lactating goats. The right (R) gland was milked thrice (3x) daily, while the left (L) gland was milked once (1x) daily for 6 weeks.

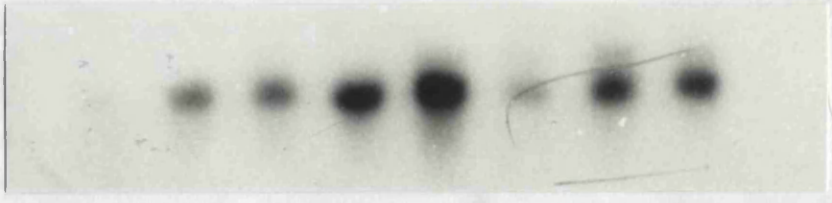
Figure 3.5 Northern analysis of α-lactalbumin mRNA before and after manipulation of milking frequency. The right gland was milked thrice daily (3x) and the left gland once daily (1x) for 6 weeks. RNA was hybridised with a caprine α-lactalbumin cDNA probe before treatment commenced and after the treatment period. Abundance of the 0.7 kb α-lactalbumin was determined by densitometry (table 3.2).

Pre-treatment

28S RNA



α-lactalbumin

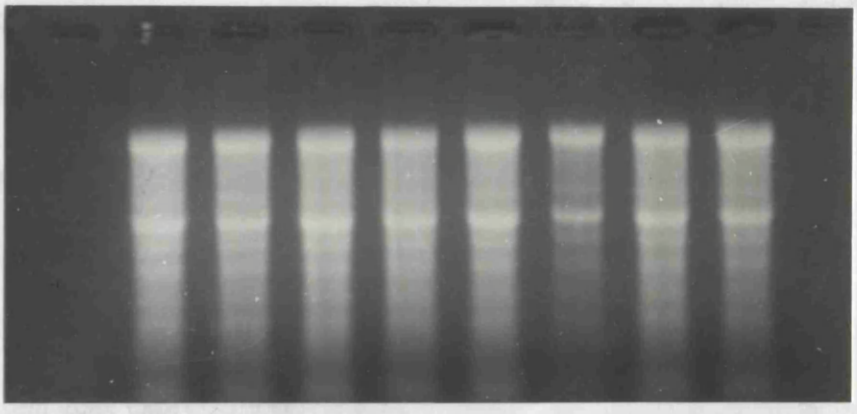


230 419 431 402

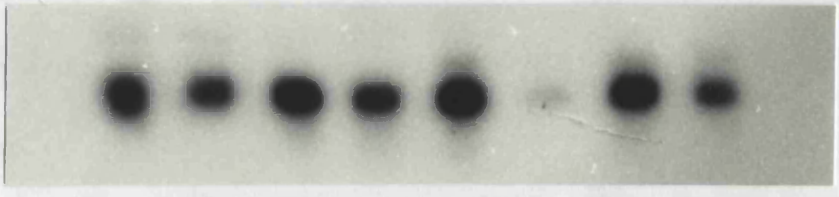
3x 1x 3x 1x 3x 1x 3x 1x

Post-treatment

28S RNA



α-lactalbumin



gland compared with the once daily milked gland (table 3.2). Indeed, over the six week treatment period, three of the four animals (230, 402 and 419) showed increased levels of α-lactalbumin mRNA/mg DNA in the thrice daily milked gland compared to pre-treatment levels in the same gland, whereas in the contralateral gland, once daily milking decreased α-lactalbumin gene expression (table 3.2). In all three animals, the increase in milk protein mRNA abundance expressed on a per cell basis (i.e. per mg DNA), was the result of a relative increase in the amount of RNA per cell in the test gland and an increase in the abundance of α-lactalbumin mRNA per unit cellular RNA (results not shown).

Casein gene expression was similarly affected (figure 3.6). Again, an increase in the frequency of milk removal led to higher levels of α_{s1}-casein mRNA in the thrice daily milked gland. As before, the more frequently milked gland of three animals (230, 402 and 419) showed an increase in α_{s1}-casein mRNA abundance during the treatment period. Comparison of pre- and post-treatment levels in the once daily milked gland indicated a decrease in the abundance of this mRNA, suggesting that once daily milking also decreased α_{s1}-casein gene expression (table 3.2).

Although there was apparently a decrease in both α-lactalbumin and α_{s1}-casein mRNA abundance in one animal (431) during the experiment, the levels in the thrice daily milked gland at the end of the experiment were markedly higher than in the once daily milked gland (table 3.2). In this animal it was not possible to determine pre-treatment

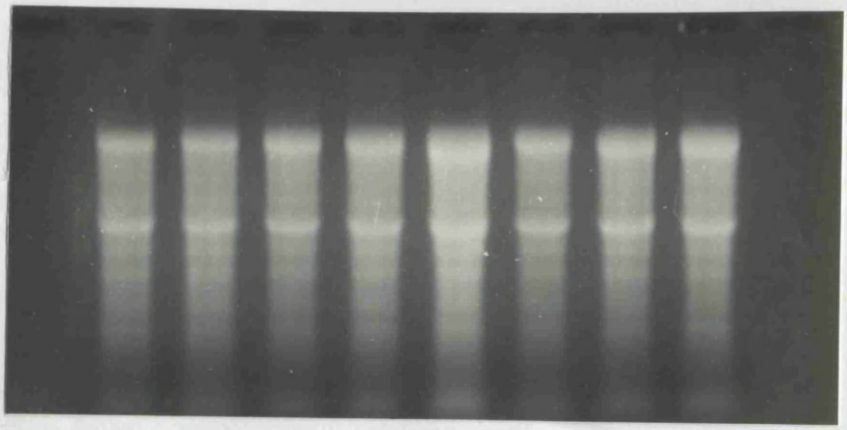
Goat	Gland	mg RNA/mg DNA		Units mRNA/mg DNA			
				α-lactalbumin		α_{s1}-casein	
		pre	post	pre	post	pre	post
230	R	1.28	0.69	270	1042	1015	1385
	L	1.68	0.68	1308	810	1660	954
419	R	1.06	0.82	764	1472	965	976
	L	0.90	0.71	1330	1081	833	841
431	R	1.82	0.88	2166	1542	5004	2608
	L	*	1.39	*	500	*	521
402	R	1.35	1.05	1468	1745	3762	4769
	L	1.44	0.88	1317	980	3068	1767

Table 3.2 α-lactalbumin and α-$_{s1}$-casein gene expression in goat mammary glands milked at different frequencies (once or thrice daily for six weeks. Total RNA was isolated from glands before and after treatment. α-lactalbumin and α_{s1}-casein mRNA abundance was determined by northern analysis and is expressed as the integrated area of the 0.7 kb α-lac mRNA and the 1.2 kb α_{s1}-cas mRNA normalised against 28S ribosomal RNA. (* indicates no value as insufficient sample for DNA assay).

Figure 3.6 Northern analysis of α_{s1}-casein mRNA before and after manipulation of milking frequency. The right gland was milked thrice daily (3x) and the left gland once daily (1x) for 6 weeks. RNA was hybridised with an ovine α_{s1}-casein cDNA probe before treatment commenced and after the treatment period. Abundance of the 1.2 kb α_{s1}-casein mRNA was determined by densitometry (table 3.3).

Pre-treatment

28S RNA



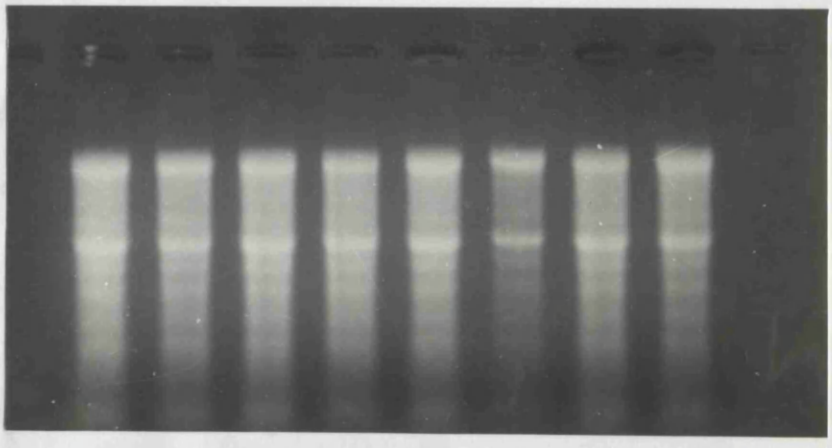
α_{s1}-casein

230 419 431 402

3x 1x 3x 1x 3x 1x 3x 1x

Post-treatment

28S RNA



α_{s1}-casein

DNA content, therefore a value for the abundance of α-lactalbumin and α_{s1}-casein mRNA could not be obtained.

Milk yield data and highly positive RMYQ values confirm the differential response of the two glands to different milking frequencies. The data also suggest a unilateral change in milk protein gene expression as a result of milking glands at different frequencies.

3.3.3 Suckling versus once daily milking

The comparison between once daily milking and suckling by kids resulted in a preferential increase in milk yield in the suckled gland (figures 3.7 & 3.8), shown as a positive RMYQ of 1.55 ± 0.16 (mean ± SEM, n = 3). This RMYQ value clearly demonstrated a differential effect of the two milking regimens. However, both treatments did in fact increase milk yield during the six week treatment period. The yield of the suckled glands increased by 196.6 ± 22% over the treatment period, whilst the yield of the once daily milked glands increased by 123.6 ± 21.4 %. Individual RMYQ values and pre- and post-treatment milk yields are shown in table 3.3.

A differential stimulation of milk yield in the suckled glands was associated with higher levels of mRNA for both α-lactalbumin and α_{s1}-casein (figure 3.9). Alpha-lactalbumin mRNA abundance, expressed on a DNA basis, was 1.3 - 2.5 fold greater in the suckled gland than in the once daily milked gland after the treatment period, while the abundance of α_{s1}-casein mRNA in the suckled gland was greater by 1.4 to 1.8 fold. As

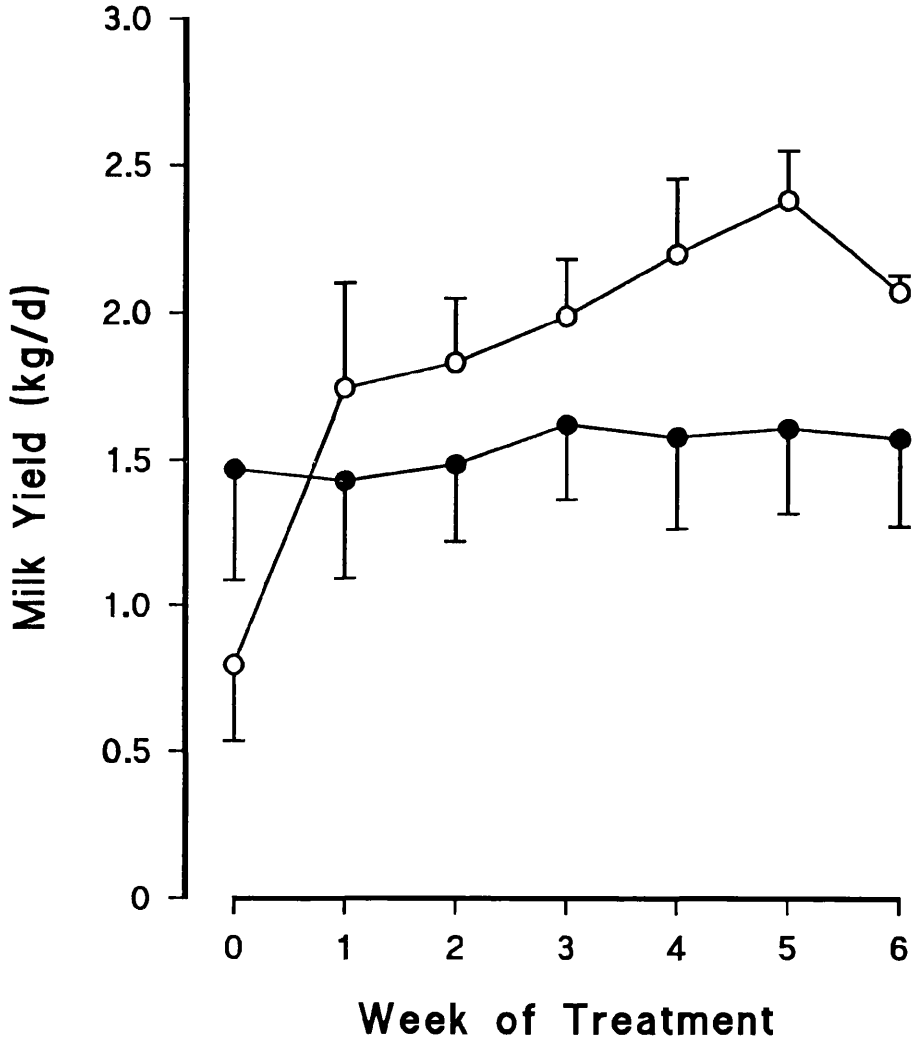
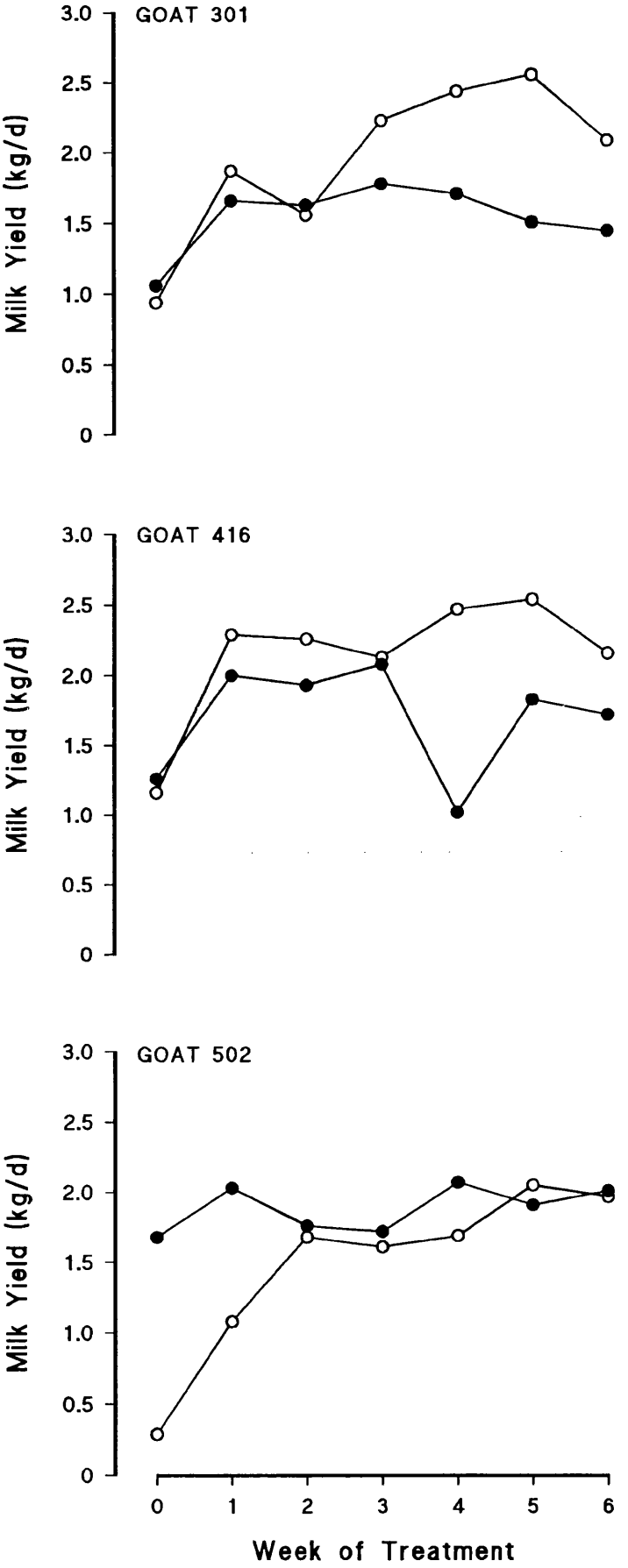


Figure 3.7 Effect of unilateral suckling on milk yield. The right gland was suckled by kids (-o-), while the left gland was milked once daily (-●-) for 6 weeks. Values are the mean ± SEM for 3 animals.

Figure 3.8 Individual gland milk yield in goats milked once daily in one gland and suckling a kid on the other gland. The right gland was suckled by kids (-o-), while the left gland was milked once daily (-●-) for 6 weeks.


GOAT 301
GOAT 416
GOAT 502
Milk Yield (kg/d)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
1
2
3
4
5
6
Week of Treatment

Goat	Gland	Treatment	Milk Yield pre (kg/d)	Milk Yield (post (kg/d)	RMYQ
301	R	S	0.94	2.09	1.62
	L	1x	1.06	1.45	
416	R	S	1.16	2.16	1.36
	L	1x	1.26	1.72	
502	R	S	1.08	1.97	1.67
	L	1x	2.03	2.01	

Table 3.3 Effect of unilateral suckling and once daily milking on individual gland milk yield in three lactating goats. The right (R) gland was suckled (S) by kids, while the left (L) gland was milked once (1x) daily for 6 weeks.

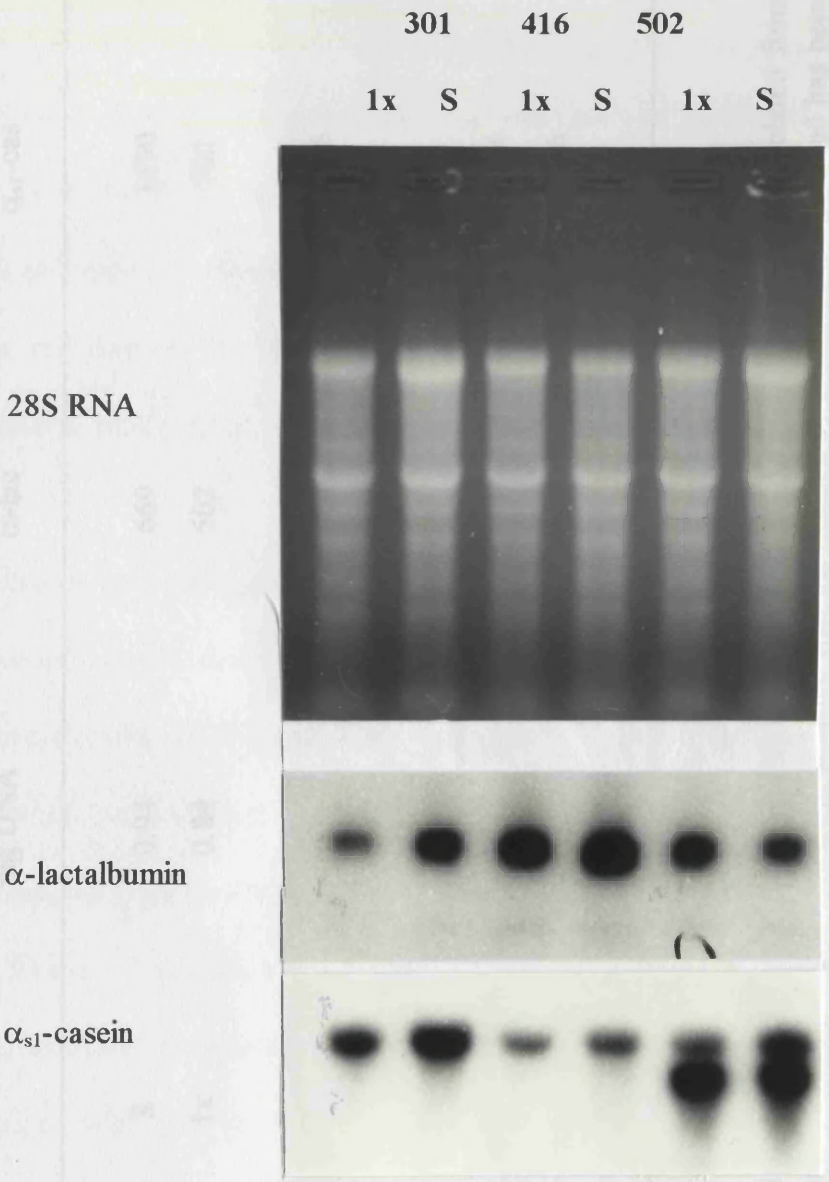


Figure 3.9 Northern analysis of RNA prepared from mammary glands milked once daily or suckled for 6 weeks. The right gland was available for suckling (S) by kids while the left gland was milked once daily (1x) for 6 weeks. RNA was hybridised with caprine α-lactalbumin and ovine α_{s1}-casein cDNA probes. Abundance of the 0.7 kb α-lactalbumin mRNA and the 1.2 kb α_{s1}-casein m RNA was determined by densitometry (table 3.5).

Goat	Gland	Treatment	mg RNA/ mg DNA	Units mRNA/mg DNA	
				α-lac	α_{s1}-cas
301	R	S	0.94	669	1190
	L	1x	0.84	502	780
416	R	S	2.14	1660	1586
	L	1x	0.56	665	677
502	R	S	1.78	2590	5764
	L	1x	0.93	678	3386

Table 3.4 Effect of suckling on α-lactalbumin and α_{s1}-casein gene expression in goat mammary gland. Total RNA was isolated from glands milked differentially for 6 weeks. Alpha-lactalbumin and α_{s1}-casein mRNA abundance was determined by Northern analysis and has been expressed as the integrated area of the 0.7 kb α-lac mRNA and the 1.2 kb α_{s1}-cas mRNA normalised against 28S ribosomal RNA.

in those animals subjected to unilateral once and thrice daily milking, the effect on milk protein gene expression again appeared to be due to an increase in the amount of RNA per cell and higher levels of α-lactalbumin and α_{s1}-casein mRNA per unit cellular RNA (results not shown) in the suckled glands, which together produced a greater abundance of milk protein mRNA (table 3.4).

3.3.4 Continuous drainage versus once daily milking

The change to once daily milking and continuous drainage of milk resulted in little difference in milk yield between the two glands (figures 3.10 and 3.11). Animals treated in this way showed a RMYQ of 0.92 ± 0.35 (mean ± SEM, n = 3), and only one animal, 417, showed a positive response in terms of milk yield, with an RMYQ of 1.32. These results were unexpected as it was anticipated that continuous milk removal from the gland would increase milk yield markedly. Experimental difficulties experienced with this method of milk removal were probably responsible for the negligible effect on milk yield. Individual RMYQ values and pre- and post-treatment milk yields are shown in table 3.5.

Continuous drainage did not result in a differential effect on milk protein gene expression (figure 3.12). As shown in table 3.6, the continuously drained gland had lower levels of α-lactalbumin and α_{s1}-casein mRNA/mg DNA than in the once daily milked gland, in two of the three animals (414 & 417). In only one goat (422) was there an increase in abundance of each mRNA compared with once daily milking.

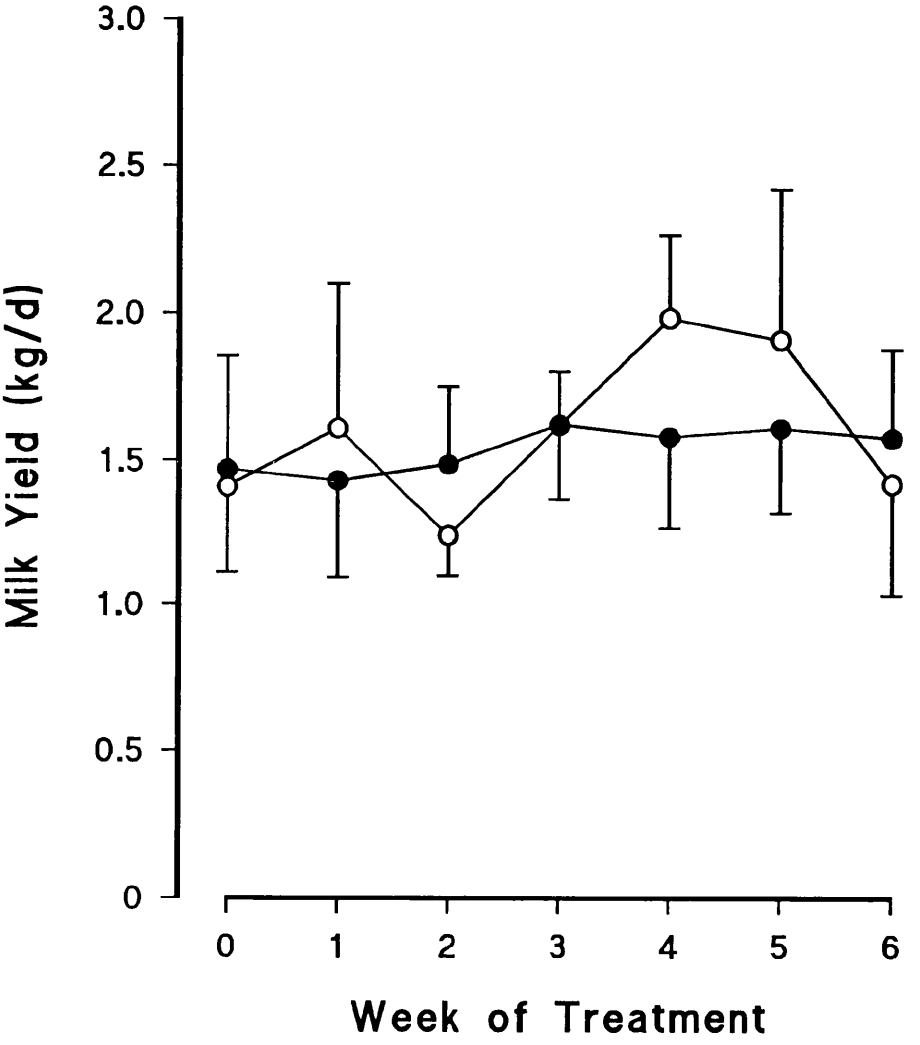
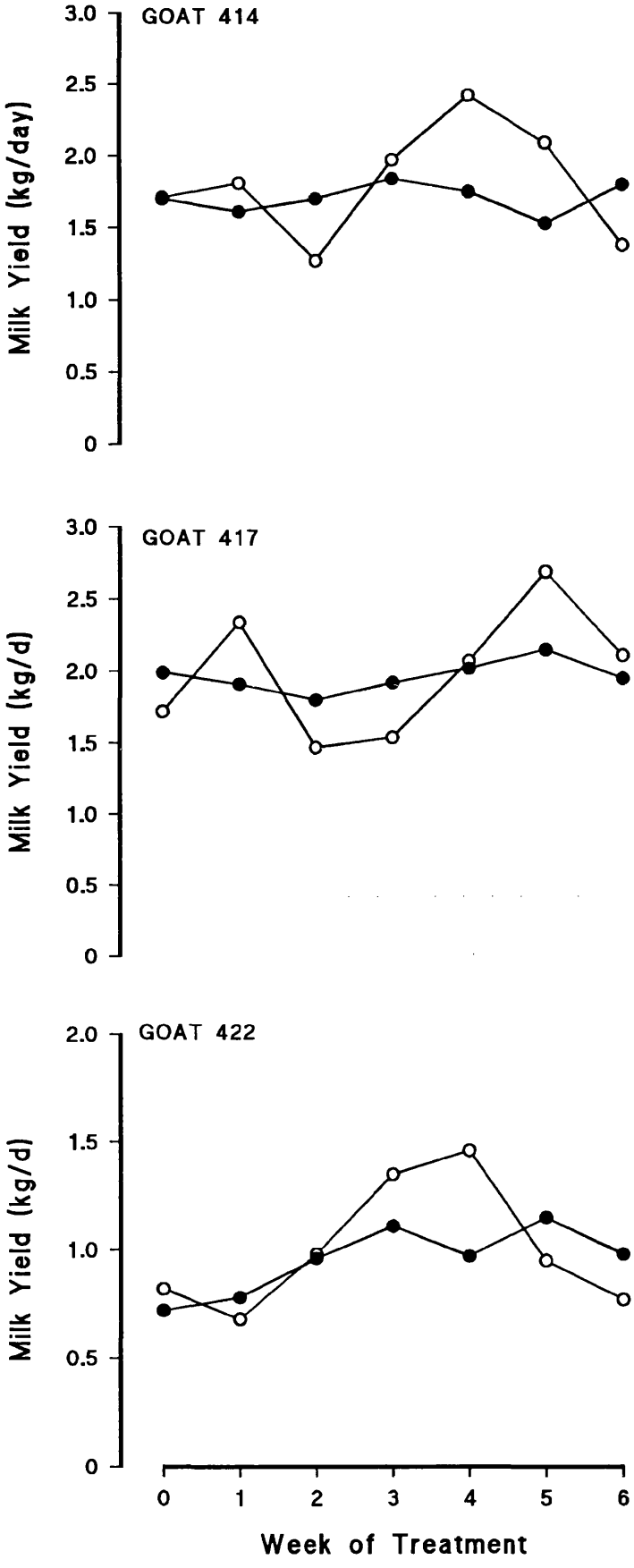


Figure 3.10 Effect of unilateral milk drainage or once daily milking on milk yield. The right gland was drained continuously (-o-), while the left gland was milked once daily (-●-) for 6 weeks. Values are the mean ± SEM for 3 animals.

Figure 3.11 Individual gland milk yield in goats subjected to once daily milking or milk drainage. The right gland was drained continuously (-o-), while the left gland was milked once daily (-●-) for 6 weeks.


GOAT 414
Milk Yield (kg/day)
3.0
2.5
2.0
1.5
1.0
0.5
0
GOAT 417
Milk Yield (kg/d)
3.0
2.5
2.0
1.5
1.0
0.5
0
GOAT 422
Milk Yield (kg/d)
2.0
1.5
1.0
0.5
0
0 1 2 3 4 5 6
Week of Treatment

Goat	Gland	Treatment	Milk Yield pre (kg/d)	Milk Yield (post (kg/d)	RMYQ
414	R	D	1.71	1.38	0.76
	L	1x	1.70	1.80	
417	R	D	1.72	2.11	1.32
	L	1x	1.99	1.95	
422	R	D	0.82	0.77	0.68
	L	1x	0.72	0.98	

Table 3.5 Effect of continuous drainage on milk yield in the goat mammary gland. The right (R) gland was continuously drained (D), while the left (L) gland was milked once (1x) daily.

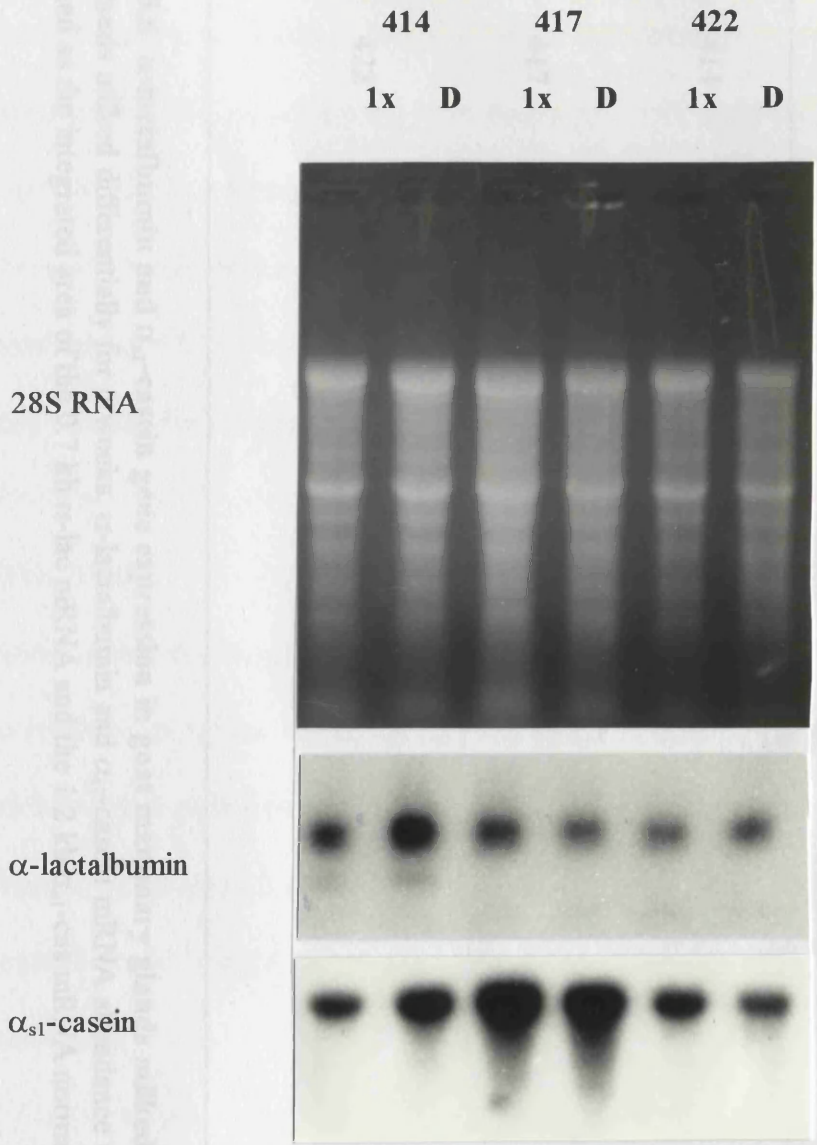


Figure 3.12 Northern analysis of RNA prepared from mammary glands milked once daily or drained for 6 weeks. The right gland was drained continuously (D) while the left gland was milked once daily (1x) for 6 weeks. RNA was hybridised with caprine α-lactalbumin and ovine α_{s1}-casein cDNA probes. Abundance of the 0.7 kb α-lactalbumin mRNA and the 1.2 kb α_{s1}-casein m RNA was determined by densitometry (table 3.7).

Goat	Gland	Treatment	mg RNA/ mg DNA	Units mRNA/mg DNA	
				α-lac	α_{s1}-cas
414	R	D	1.01	1808	3542
	L	1x	1.16	2070	3868
417	R	D	0.96	1106	1032
	L	1x	0.81	1133	1700
422	R	D	1.73	2862	2729
	L	1x	1.14	1247	1680

Table 3.6 α-lactalbumin and α_{s1}-casein gene expression in goat mammary glands milked once daily or drained. Total RNA was isolated from glands milked differentially for 6 weeks. α-lactalbumin and α_{s1}-casein mRNA abundance was determined by Northern analysis and has been expressed as the integrated area of the 0.7 kb α-lac mRNA and the 1.2 kb α_{s1}-cas mRNA normalised against 28S ribosomal RNA.

In this study continuous drainage of the gland was demonstrated to be ineffective and in consequence no consistent effect of this method of milk removal on milk yield was observed. Similarly no consistent difference in α-lactalbumin and α_{s1}-casein gene expression was observed between once daily milked and drained glands at the end of the experiment. Therefore, local stimulation of mammary gene expression clearly depended on effective removal of milk from the gland.

3.4 DISCUSSION

This series of experiments investigated the effects of both short (9 days) and longer term (6 weeks) alterations in milking frequency on milk yield and milk protein gene expression in the lactating goat mammary gland. The results showed that manipulation of milking frequency and concomitant changes in the rate of milk secretion are accompanied in the long term, but not in the short term, by changes in milk protein gene expression.

Acute regulation of milk secretion by milk removal is well documented. The current study confirms that milking one gland of goats more frequently stimulates milk yield unilaterally (Henderson *et al.*, 1983; Wilde & Knight, 1990). Unilateral thrice daily milking increased milk yield over and above any change in the yield of the once daily milked gland. Early experiments eliminated the possibility that this local effect was due to systemic factors (Linzell & Peaker, 1971; Blatchford *et al.*, 1982), or that it reflected changes in gland distension by stored milk (Henderson & Peaker, 1984). Instead it has been shown to be due to withdrawal of a milk constituent (Henderson &

Peaker, 1984) which has been identified as a small milk protein termed FIL or Feedback Inhibitor of Lactation (Addey *et al.*, 1991b; Wilde *et al.*, 1995).

In the present study, there was considerable variation between pre- and post-treatment milk yield values and in the response to frequent milking, frequent milk removal or suckling. It is possible that these variations were due to differences in gland anatomy which affect each gland's susceptibility to autocrine inhibition by FIL. Previous studies have demonstrated that the response to milking frequency depends on the site of milk storage within the gland: animals which store a high proportion of their milk in the alveoli show a greater response to more frequent milking (Knight *et al.*, 1989). Conversely, animals with large cisternal storage are more tolerant of once daily milking and less responsive to thrice daily milking (Dewhurst & Knight, 1992). These observations are consistent with the theory that FIL acts via the apical surface of the mammary secretory cell to regulate the rate of milk secretion (Henderson & Peaker, 1984; Wilde *et al.*, 1995).

In contrast to the effect of frequent milking, when milk removal was by catheter drainage, yield was not affected. This result was surprising since this method of milk removal was expected to increase the rate at which milk was removed from the gland. However, catheters do not remove alveolar milk, therefore catheter drainage, in not evacuating the alveoli of the gland, may have failed to relieve autocrine inhibition and therefore would not be expected to elicit a stimulation of milk secretion (Henderson & Peaker, 1987). The effect of catheter drainage on milk yield may also have been due to

difficulties in maintaining continuous milk removal. The teat cannulas used to facilitate this method frequently became blocked or dislodged, leading to discontinuities in the rate of milk removal.

When frequent and effective milk removal was maintained for an extended period, as in thrice daily milked and suckled glands, it was apparent that the persistent stimulation of milk secretion was sustained by changes in milk protein gene expression. As with changes in milk yield, effects on α-lactalbumin and α_{s1}-casein mRNA were apparent as differential responses in the glands subjected to different milking regimes, indicating that these changes were elicited locally within each mammary gland. In contrast, when manipulation of milk removal was not successful in regulating milk yield, such as in the continuously drained glands, milk protein gene expression was not affected. Therefore, changes in milk protein gene expression, like changes in milk yield, are dependent on effective manipulation of milk removal.

Acute regulation of milk secretion by changes in milking frequency appears to be independent of developmental adaptations in the tissue. In the short term, increased output of milk proteins was not dependent on an increase in their mRNA abundance (this study; Bryson *et al.*, 1993). In contrast, sustained manipulation of milk removal regulates mammary differentiation (Wilde *et al.*, 1987): frequent milk removal for several weeks elicited significant increases in the activities of several key enzymes involved in milk synthesis (Wilde *et al.*, 1987). Increases in the activities of key enzymes such as fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) were

associated with an increase in mRNA abundance for these proteins (Barber & Travers, 1993; Wilde *et al.*, 1990). In the present study we have now demonstrated that this local regulation of mammary gene expression, illustrated first by the lipogenic enzymes FAS and ACC, extends to milk protein genes, as exemplified by the increase in α-lactalbumin and α_{s1}-casein mRNA abundance.

The increase in the abundance of α-lactalbumin and α_{s1}-casein mRNA observed following extended periods of more frequent milk removal may be due to either an increase in transcription rate, an increase in mRNA stabilisation, or a combination of both. In each case these changes are hormone dependent, and are particularly dependent on galactopoietic hormones such as prolactin (Matusik & Rosen, 1978; Teyssot & Houdebine, 1980). How the differential changes were elucidated in the lactating goat is not clear, but one possibility is that this is a consequence of local modulation of the cells sensitivity to circulating hormones by up or down regulation of cell surface hormone receptors (Wilde *et al.*, 1990). Increases in milking frequency have been associated with increases in the number of prolactin receptors in the more frequently milked gland (McKinnon *et al.*, 1988). A whey fraction containing FIL has been found to down-regulate prolactin receptors in primary cell culture (Bennett *et al.*, 1990), and this effect has recently been reproduced using purified FIL protein (CN Bennett, 1993). Differential changes in prolactin sensitivity elicited by local modulation of autocrine feedback may therefore be competent to elicit unilateral changes in secretory cell differentiation and local modulation of milk protein gene expression.

Chapter Four

Autocrine regulation of mammary gene expression in primary culture

4.1 INTRODUCTION

In chapter three we demonstrated that local changes in milk secretion and mammary gene expression are modulated by frequency of milking. Evidence from cell culture and *in vivo* experiments indicates that the acute response is due to feedback inhibition by FIL, a secreted milk protein. FIL, or the feedback inhibitor of lactation, is synthesised by the mammary epithelial cell and secreted into the alveolar lumen along with other milk constituents (Wilde *et al.*, 1995). FIL has been shown to decrease milk constituent synthesis in rabbit mammary tissue explants (Wilde *et al.*, 1987; Wilde *et al.*, 1995) and in suspensions of lactating murine epithelial cells (Rennison *et al.*, 1993). In lactating goats introduction of FIL into the teat duct decreased milk yield (Wilde *et al.*, 1988, 1995). Additionally, auto-immunisation of lactating goats against their own inhibitory protein was found to stimulate milk secretion and protect against the effect of once daily milking (Wilde *et al.*, 1996).

Long term increases in milking frequency are accompanied by developmental responses which act to sustain the secretory response and, like the acute response of milk secretion, occur locally in the frequently milked gland(s). The response elicited depends on the duration of the stimulus. After days to weeks, secretory cell differentiation is regulated: thrice instead of twice daily milking of one gland produced unilateral increases in key mammary enzyme activity and mRNA abundance (Travers & Barber, 1993; Wilde *et al.*, 1987b). In the long term, the same manipulation increased mammary cell number (Knight *et al.*, 1990; Wilde *et al.*, 1987b). Similarly, the

decrease in cell differentiation in glands milked infrequently or incompletely is accompanied by a net decrease in prolactin binding (McKinnon *et al.*, 1988). FIL's effect on membrane trafficking may also influence mammary cell hormone receptors and thus cell differentiation. In short, the protein's unique mechanism of action raises the possibility that it may be able to influence not just the secretory pathway, but also cause the developmental adaptations which act to sustain the response of the gland to more frequent milk removal. To investigate whether FIL influences mammary gene expression it is necessary to have available an *in vitro* system in which milk protein genes are expressed and which responds to known regulators of mammary gene expression.

Mammary epithelial cells grown on plastic do not maintain a differentiated morphology even in the presence of lactogenic hormones (Li *et al.*, 1987). In contrast, cells on floating collagen gels display enhanced milk protein synthesis and secretion in response to lactogenic hormones (Emerman *et al.*, 1977, Lee *et al.*, 1984, 1985). Murine cells cultured in this way expressed mRNA for β-casein (Li *et al.*, 1987) but not for whey acidic protein (Chen & Bissell, 1989), indicating that differentiation was not complete. More complex substrata prepared from whole mammary glands (Wicha *et al.*, 1982; Wilde *et al.*, 1984; Blum *et al.*, 1987) allowed greater functional differentiation of cultured cells. A more convenient alternative to these was subsequently found to be a reconstituted basement membrane matrix derived from the Engelbreth-Holm-Swarm tumour. This extract (EHS matrix) contains several basement membrane constituents including collagen type IV, laminin, entactin and heparan proteoglycans (Kleinman *et*

al., 1983, 1986). The influence of the extracellular matrix is due to a combination of these components, the most important of which is laminin (Streuli, 1993). The expression of milk proteins is higher on EHS matrix compared with collagen and plastic (Li *et al.*, 1987), and WAP is only expressed in EHS culture, suggesting that cells cultured on EHS have a greater differentiated function (Chen & Bissell, 1989). In addition, mouse mammary cells cultured on EHS form multicellular structures termed "mammospheres" which structurally and functionally resemble alveoli in lactating tissue (Aggeler *et al.*, 1991; Barcellos-Hoff *et al.*, 1989; Chen & Bissell, 1987, 1989; Hurley *et al.*, 1994; Li *et al.*, 1987). Mammary epithelial cells exist in the differentiated gland as a single layer of polarised cells surrounding a lumen. The major milk proteins are synthesised in the epithelial cells and secreted into the alveolar lumen under the influence of galactopoietic hormones (Topper & Freeman, 1990). Cells recruited into mammosphere behave similarly, secreting milk proteins vectorially into a central luminal space.

The EHS culture system has been used primarily for the culture of murine mammary epithelial cells. Ruminant cell culture has been limited to culture on floating collagen gels (Hansen & Knudsen, 1991; Talhouk *et al.*, 1990), and while cells cultured in this way synthesise and secrete milk proteins they do not form the polarised structures typical of alveoli *in vivo*, or attain the same degree of differentiated function. However, recent studies indicate that goat mammary epithelial cells behave similarly to their murine counterparts when cultured on EHS matrix, forming polarised three

dimensional alveolar-like structures and secreting milk proteins vectorially (LMB Finch, personal communication).

In view of their potential advantages in terms of mammary protein gene expression, these goat mammosphere cultures were used to investigate if FIL could account for the local response of gene expression to milking frequency seen *in vivo* (chapter three). To this end, goat mammary epithelial cells were cultured on EHS matrix in the presence and absence of caprine FIL after differentiation of the cells had taken place.

4.2 RESULTS

4.2.1 Mammosphere formation

Cells were plated on EHS matrix as clumps of 10 - 50 cells, while minimising the number of single cells. Within 24 h the majority of cells reorganised into small multicellular spheres (figure 4.1 A & B) which structurally resemble those assumed by murine mammary epithelial cells (Barcellos-Hoff *et al.*, 1989). Where cells clumps began to pull the matrix around themselves, distinct patterns or stress lines could be seen (figure 4.1 C). These stress lines are characteristic of murine mammary cultures on EHS matrix (Neville *et al.*, 1991). By day 3 of culture (figure 4.1 D), the multicellular structures were enshrouded in matrix, and by day 6 no individual cells were visible and the mammospheres had well-defined edges (figure 4.1 E & F). Scanning electron microscopy confirmed that cell clumps became completely enshrouded in matrix (figure 4.2). Previous studies demonstrated by immunofluorescence that goat mammary epithelial cells cultured in this way secreted

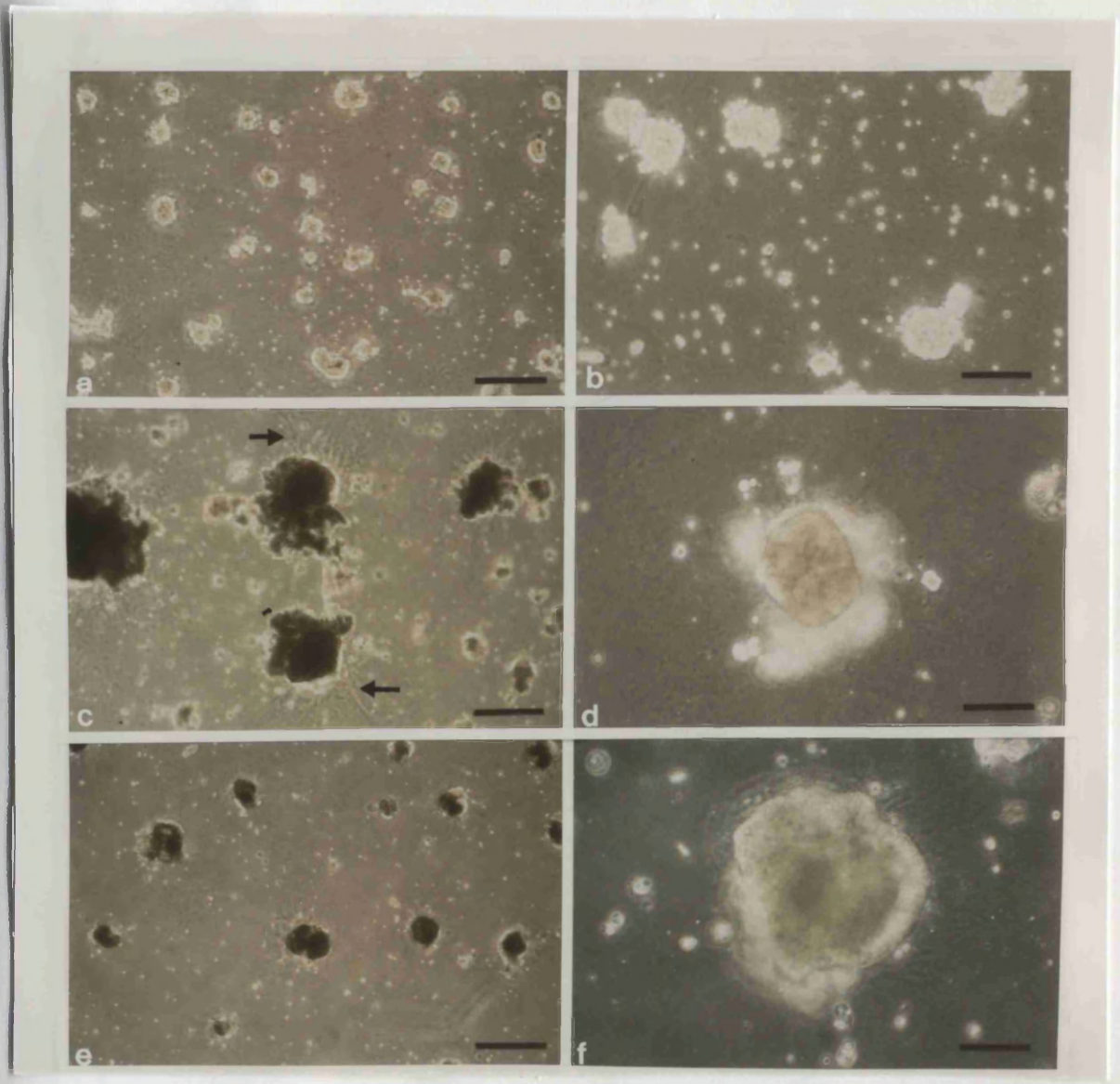


Fig. 4.1 Morphology of goat mammary epithelial cells cultured on EHS matrix.

A-F, phase contrast light micrographs. **A**, day 1, bar = 9 μm; **B**, day 1, bar = 1.4 μm; **C**, day 3, bar = 9 μm; **D**, day 3, bar = 0.95 μm; **E**, day 6, bar = 9 μm; **F**, day 6, bar = 0.95 μm. Arrows indicate stress lines (**C**).

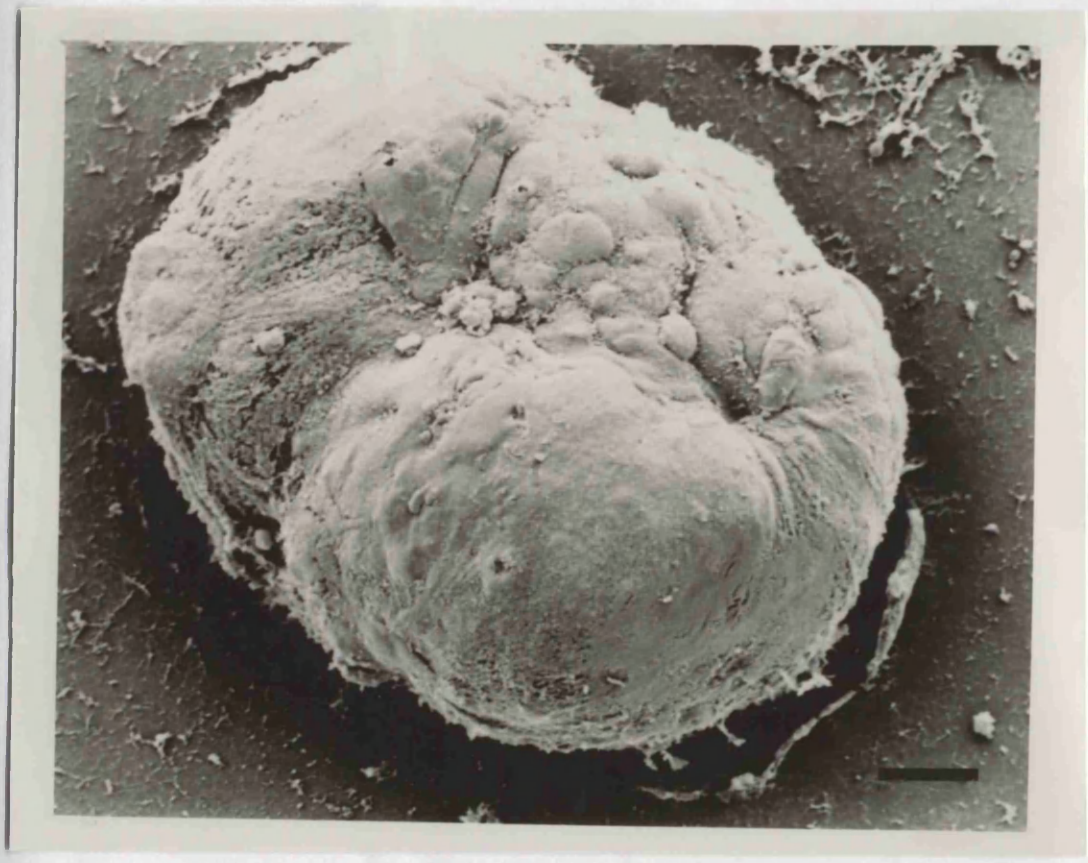


Fig. 4.2 Morphology of goat mammary epithelial cells cultured on EHS matrix. Cells were cultured for 7 days and then processed for electron microscopy. Scanning electron micrograph of a single mammosphere, bar = 9.25 μm. Micrograph courtesy of Drs LMB Finch and KAK Hendry.

α-lactalbumin and caseins into the alveolar lumen (LMB Finch, personal communication).

Plating density was important in optimising cell differentiation. For example RNA yield decreased when plating density increased. Protein secretion was similarly affected at high cell density (LMB Finch, personal communication). This may reflect a limit to mammosphere formation at any matrix concentration.

4.2.2 Milk protein gene expression

Total RNA was isolated from goat mammary epithelial cells grown on EHS matrix after 2, 5 and 7 days of culture. RNA was isolated as described in section 2.4.9. Northern blot analysis (section 2.2.4) with cDNA probes for the milk protein genes α_{s1}-casein, β-lactoglobulin (figure 4.3) and α-lactalbumin (results not shown) indicated that levels of these genes were low at day 2 and increased to a maximum at day 5 (figure 4.3). By day 7 of culture abundance of the milk protein mRNAs had decreased slightly. Throughout this time course experiment α-lactalbumin was expressed at lower levels than α_{s1}-casein, reflecting the relative abundance of each mRNA within the mammary gland. Cells cultured on plastic did not express mRNA for α_{s1}-casein, β-lactoglobulin (figure 4.3) or α-lactalbumin, demonstrating that an extracellular matrix is required for functional differentiation (figure 4.3). These results compare favourably with those seen in murine epithelial cells. Mouse cells cultured on EHS matrix express high levels of the milk protein genes β-casein (Li *et al.*, 1987), transferrin (Chen & Bissell, 1987) and whey acidic protein (Chen & Bissell, 1989). Further, Aggeler *et al.*

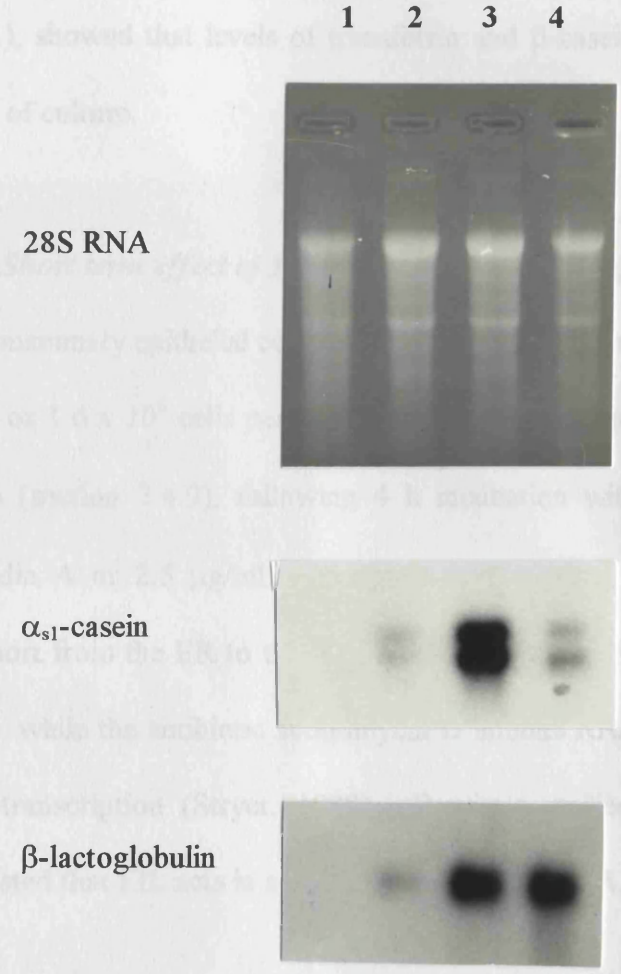


Fig. 4.3 Milk protein mRNA levels in cells cultured on EHS matrix (lanes 2 - 4) and on plastic (lane 1). Total RNA was isolated from mammary epithelial cells after 2, 5 or 7 days in culture. Northern blot analysis indicated levels of the milk protein genes α_{s1}-casein and β-lactoglobulin were maximal at day 5.

(1991), showed that levels of transferrin and β-casein mRNA remained high through day 6 of culture.

4.2.3 Short term effect of FIL on mammary gene expression

Goat mammary epithelial cells were cultured on EHS matrix for 6 days at densities of 8 x 10^5 or 1.6 x 10^6 cells per well. Total RNA was isolated from the cultured cells on day 6 (section 2.4.9), following 4 h incubation with either 8 μg/ml FIL, 5 μg/ml brefeldin A or 2.5 μg/ml actinomycin D. The drug brefeldin A is known to block transport from the ER to the Golgi (Misumi *et al.*, 1986; Lippincott-Schwartz *et al.*, 1989), while the antibiotic actinomycin D inhibits RNA chain elongation and therefore gene transcription (Stryer, 1988). Previous studies (Rennison *et al.*, 1993) have suggested that FIL acts in a similar way to brefeldin A, blocking ER to Golgi transport.

The effect of caprine FIL on milk protein gene expression was determined by northern blot analysis. Alpha-lactalbumin and α_{s1}-casein mRNA abundance was expressed as the integrated area of the 0.7 kb α-lactalbumin mRNA and 1.2 kb α_{s1}-casein mRNA, normalised against 28S ribosomal RNA. Milk protein gene expression was expressed as arbitrary units of mRNA abundance per 10 μg RNA.

In the first experiment cells were cultured at a density of 1.6 x 10^6 cells per well. Northern blot analysis indicated that 4 h exposure to FIL did not decrease the abundance of either α-lactalbumin or α_{s1}-casein mRNA (figure 4.4 A). Densitometry confirmed these observations (table 4.1). Short term exposure to FIL in these

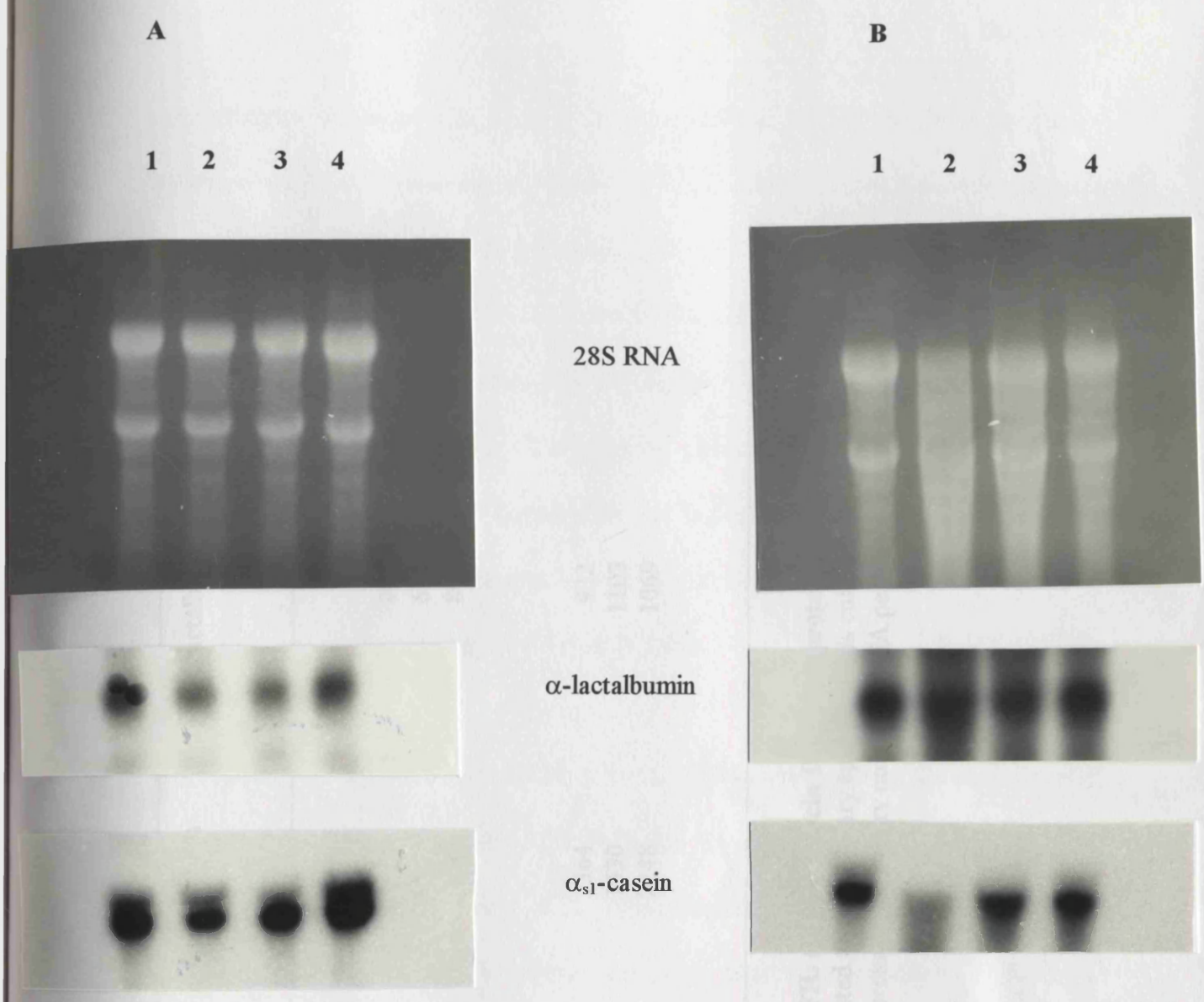


Fig. 4.4 Milk protein mRNA levels in mammary cells cultured on EHS matrix for 6 days at densities of 1.6 x 10^6 (A) or 8 x 10^5 (B) cells per well. Total RNA was isolated on day 6 of culture following 4 h incubation with 8 μg/ml FIL, 2.5 μg/ml brefeldin A or 2.5 μg/ml actinomycin D. Lane 1 control, 2 actinomycin D, 3 brefeldin A, 4 FIL.

Experiment	Treatment	α_{s1}-casein	α-lactalbumin
1	Control	1518	932
	Actinomycin D	1155	618
	FIL	2065	808
2	Control	1364	932
	Actinomycin D	1530	1103
	FIL	2248	1069

Table 4.1 Effect of short term exposure to FIL or actinomycin D on milk protein gene expression in mammosphere culture. Total RNA was isolated from mammary epithelial cells cultured on EHS matrix for 6 days (see text for details). Results are expressed as arbitrary units of mRNA per 10 μg of RNA.

circumstances increased the abundance of α_{s1}-casein mRNA by 36%, while α-lactalbumin mRNA abundance decreased by 13.3%. In the second experiment, cells were grown on EHS matrix at a density of 8 x 10^5 cells per well. Northern blot analysis again indicated that 4 h exposure to FIL did not decrease the abundance of either α-lactalbumin or α_{s1}-casein mRNA (figure 4.4 B). Densitometry confirmed these observations (table 4.1). Under these circumstances 4 h exposure to FIL increased the abundance of α-lactalbumin and α_{s1}-casein mRNA by 15% and 64% respectively. Exposure to actinomycin D had no consistent effect on the abundance of either milk protein mRNA (figure 4.4 A & B; table 4.1).

Short-term (4 h) exposure to brefeldin A had no consistent effect on the abundance of either α-lactalbumin or α_{s1}-casein mRNA (figure 4.4 A & B). Densitometric analysis confirmed this observation (table 4.2). Two further experiments also showed no consistent effect on milk protein mRNA abundance after 4 h exposure to brefeldin A. Treatment of cells with this fungal drug decreased α_{s1}-casein and α-lactalbumin mRNA abundance (figure 4.7) but increased the abundance of β-lactoglobulin mRNA (figure 4.8). The results of this series of experiments are summarised in table 4.2.

The increase in α_{s1}-casein mRNA abundance in FIL treated cells was unexpected, and not shown by other milk proteins. To explore further FIL's effect on message abundance goat mammary epithelial cells were cultured with FIL for longer time periods (sections 4.2.4 and 4.2.5).

Experiment	Treatment	α_{s1}-casein	α-lactalbumin	β-lactoglobulin
1	Control	1518	932	-
	Brefeldin A	1393	614	-
2	Control	1364	932	-
	Brefeldin A	2108	991	-
3	Control	4132	2835	-
	Brefeldin A	3472	2046	-
4	Control	3255	-	866
	Brefeldin A	4231	-	637

Table 4.2 Effect of 4 h exposure to brefeldin A on milk protein gene expression in mammosphere culture. Total RNA was isolated from mammary epithelial cells cultured on EHS for 6 days (see text for details). Results are expressed as arbitrary units of mRNA per 10 μg RNA.

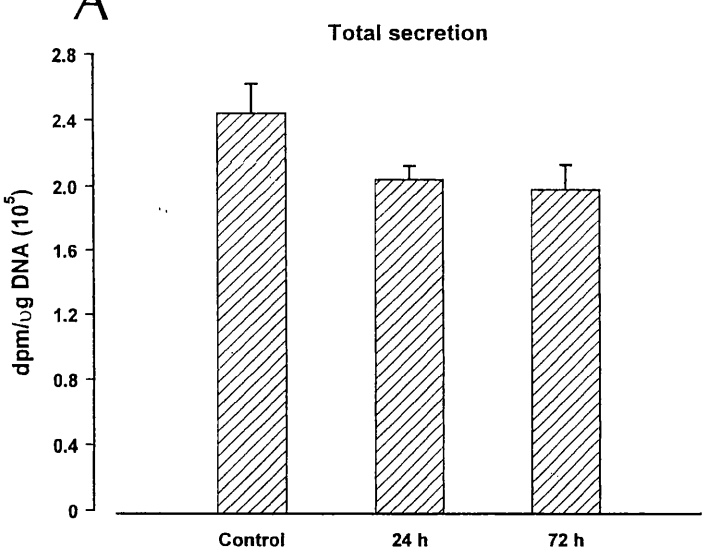
4.2.4 Effect of FIL on protein synthesis and secretion

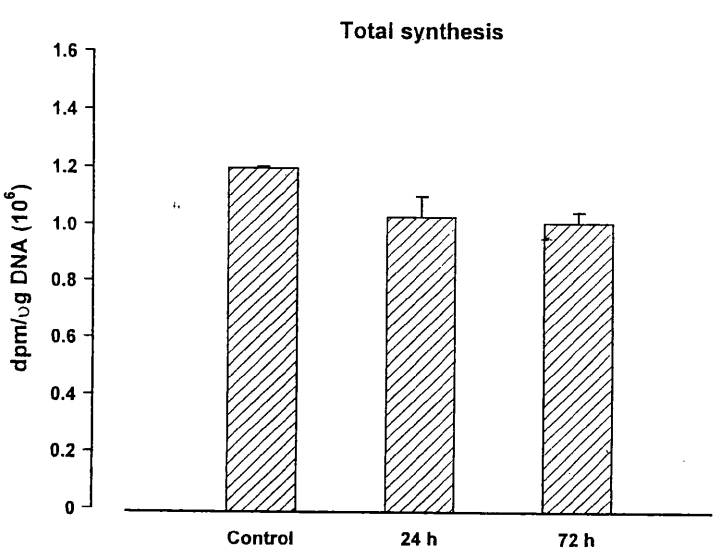
Goat mammary epithelial cells cultured on EHS matrix in the presence and absence of FIL were labelled with [^{35}S]-methionine to determine their synthetic and secretory activity (section 2.4.10.). Total protein synthesis was estimated by incorporation of radioactivity into TCA-precipitable protein in cells, culture medium and mammosphere lumina (2.4.11). In mammosphere culture, milk proteins are preferentially secreted into the lumen, which is a sealed area. EGTA may be used to open intercellular tight junctions transiently, allowing collection of lumenal contents. Collection of this medium, or EGTA fraction, allowed estimation of total protein secreted by cells in culture.

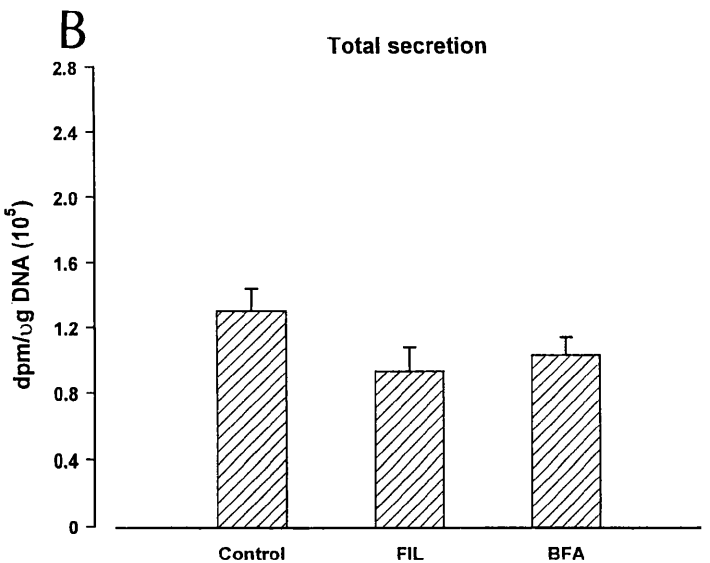
Two experiments were carried out. In the first, goat mammary epithelial cells were cultured with 8 μg/ml caprine FIL for the last 24 or 72 h of culture. In a second experiment cells were exposed to 8 μg/ml FIL or 8 μg/ml whey protein for the last 72 h of culture, or with 5 μg/ml brefeldin A for the final 4 h of culture.

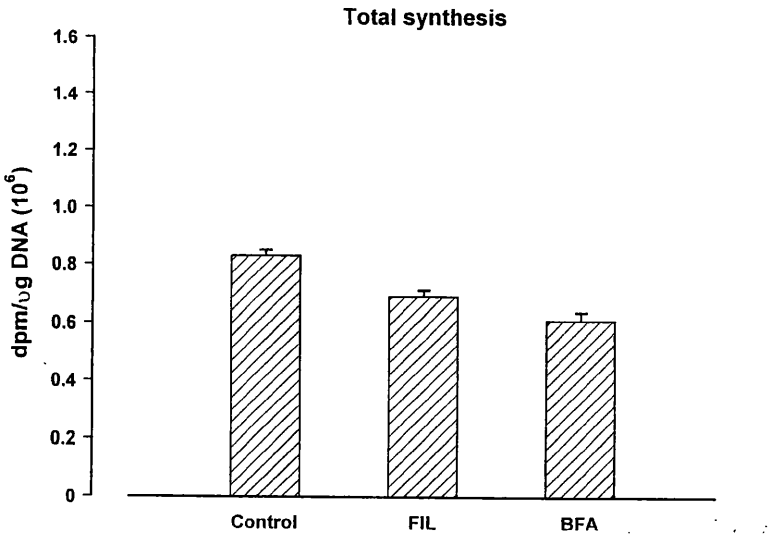
The effects of these treatments on protein synthesis and secretion by mammary epithelial cells in culture are shown in figure 4.5. Total protein synthesis and secretion after 24 or 72 h exposure to FIL is shown in panel A, while panel B summarises the consequence of 72 h exposure to FIL, or 4 h exposure to brefeldin A. No results for protein synthesis and secretion after incubation with whey protein are shown due to technical difficulties. Total protein synthesis and secretion decreased by 15.1% and 16.4% respectively after 24 h treatment with FIL. Following 72 h exposure to FIL

Fig. 4.5 Protein synthesis and secretion in mammary cells cultured on EHS matrix for 6 days. Cells were labelled with L-[^{35}S] methionine for a 4 h period following 24 or 72 h incubation with 8 μg/ml FIL (A) or following 72 h incubation with 8 μg/ml FIL, 8 μg/ml whey protein or 4 h incubation with 5 μg/ml brefeldin A (B). Total protein synthesis and secretion were measured as TCA-precipitable radioactivity in cells plus medium and in culture medium respectively. Values are the mean ± SEM for three replicate measurements at each time point.


A
Total secretion
dpm/µg DNA (10^5)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
Control
24 h
72 h


Total synthesis
dpm/µg DNA (10^6)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
24 h
72 h


B
Total secretion
dpm/µg DNA (10^5)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
Control
FIL
BFA


Total synthesis
dpm/µg DNA (10^6)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
FIL
BFA

protein synthesis fell by 16.4%, while secretion decreased by 18.8% (figure 4.5 A). Similarly, in the second experiment, exposure to FIL decreased synthesis and secretion by 17.2% and 28.5%, respectively. Brefeldin A treatment was responsible for a 26.4% decrease in protein synthesis and a reduction in secretory activity of 20.2% (figure 4.5 B).

In summary 24 or 72 h exposure to 8 μg/ml FIL reduced both protein synthesis and secretion in goat mammary epithelial cells cultured on EHS matrix. Brefeldin A, in decreasing protein synthesis and secretion, affected mammosphere cultures in a manner similar to the effect of this fungal drug observed in mouse mammary acini culture (Rennison *et al.*, 1993).

4.2.5 Effect of FIL on mammary gene expression in long term culture

In this series of experiments goat mammary cells were incubated with 8 μg/ml caprine FIL for extended periods (24 h or 72 h). Total RNA was isolated on day six of culture after treatment with FIL. The influence of FIL on gene expression in longer term cultures was analysed by northern blot analysis. Once again, α_{s1}-casein and α-lactalbumin mRNA abundance was expressed as the integrated area of the 0.7 kb α-lactalbumin mRNA and the 1.2 kb α_{s1}-casein mRNA, normalised against 28S ribosomal RNA. Beta-lactoglobulin mRNA abundance was expressed as the integrated area of the 0.9 kb β-lactoglobulin mRNA respectively, normalised against 28S ribosomal RNA.

Three experiments were carried out. In the first, goat mammary epithelial cells were cultured with 8 μg/ml caprine FIL for the last 24 or 72 h of culture. In both the second and third experiments, cells were cultured with 8 μg/ml FIL or 8 μg/ml whey protein for the last 72 h of culture.

In the first experiment where cells were exposed to FIL for 24 or 72 h, northern blot analysis (figure 4.6) indicated that α_{s1}-casein and α-lactalbumin gene expression decreased following 24 and 72 h incubation with FIL. Densitometric analysis confirmed these observations (table 4.3). Alpha-lactalbumin mRNA abundance after 24 or 72 h exposure to FIL was 31.1% and 51.7% less, respectively, of message abundance in untreated cells. Casein gene expression was similarly affected. The abundance of α_{s1}-casein mRNA decreased by 37.5% compared to untreated cells after 24 h treatment with FIL, and 72 h treatment decreased abundance by 63.3%.

This initial experiment suggested that FIL influenced milk protein gene expression. In a second experiment, goat mammary cells were incubated with 8 μg/ml FIL or 8 μg/ml caprine whey protein for 72 h. Total RNA was isolated on day six of culture following these treatments. Seventy-two hours exposure to FIL decreased both α_{s1}-casein and α-lactalbumin gene expression (figure 4.7). Northern blot analysis also indicated treatment with whey decreased expression of these milk protein genes. Densitometry confirmed these observations (table 4.2). Exposure to FIL decreased α_{s1}-casein mRNA abundance by 15.5%, while α-lactalbumin mRNA abundance decreased by 34.1%, compared to mRNA abundance in untreated cells. A decrease in mRNA abundance for

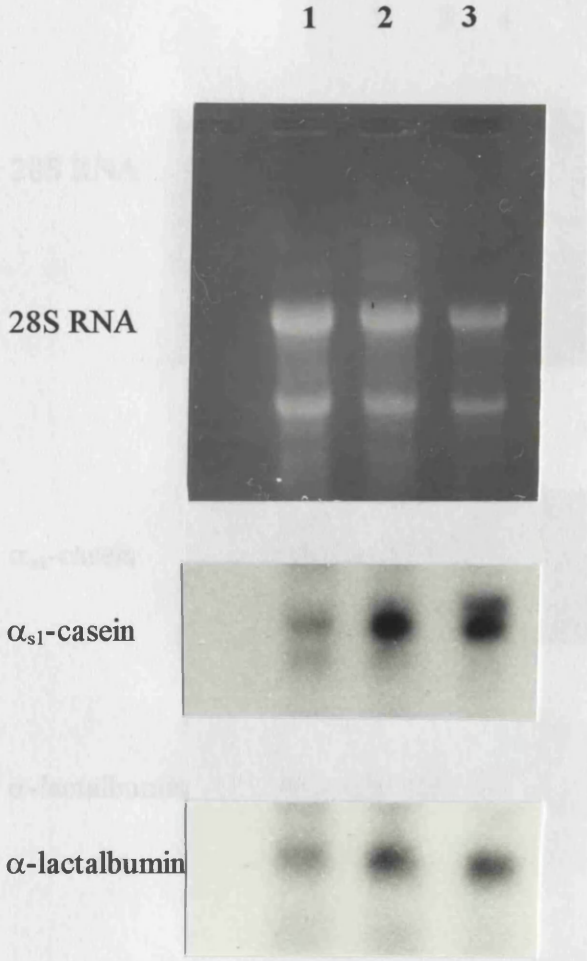


Fig. 4.6 Milk protein mRNA levels in mammary cells cultured on EHS matrix for 6 days. Total RNA was isolated on day 6 of culture following 24 or 72 h incubation with 8 μg/ml FIL. Lane **1**, 72 h FIL; lane **2**, 24 h FIL; lane **3**, control.

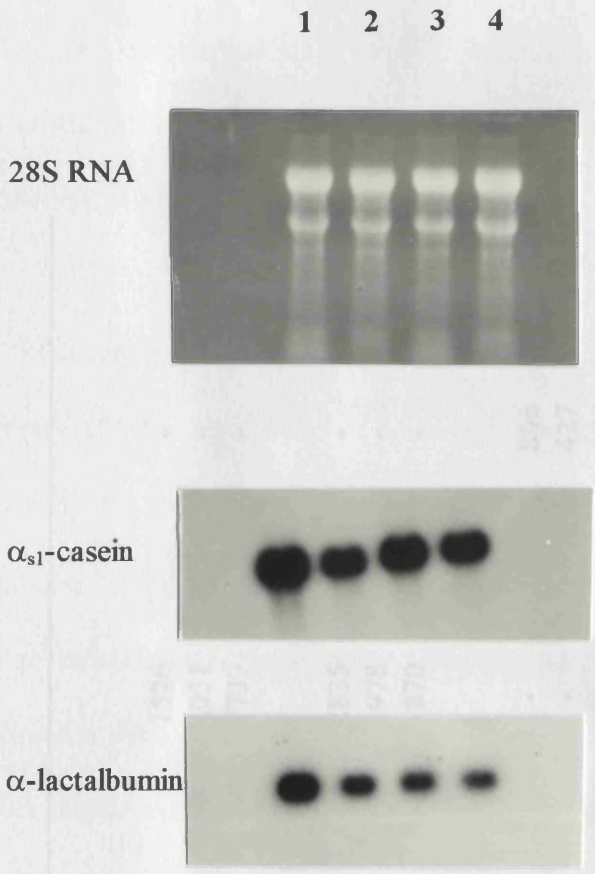


Fig. 4.7 Milk protein mRNA abundance in mammary cells cultured on EHS matrix for 6 days. Total RNA was isolated following 72 h incubation with 8 μg/ml FIL or 8 μg/ml total whey protein, or 4 h incubation with 5 μg brefeldin A. Lane **1**, control; lane **2**, 4 h brefeldin A; lane **3**, 72 h whey protein; lane **4**, 72 h FIL.

Experiment	Treatment	α_{s1}-casein	α-lactalbumin	β-lactoglobulin
1	Control	2730	1526	-
	FIL - 24 h	1707	1051	-
	FIL - 72 h	1003	737	-
2	Control	4132	2835	-
	Whey	3956	1978	-
	FIL	3495	1870	-
3	Control	3255	-	866
	Whey	2240	-	427
	FIL	1551	-	397

Table 4.3 Effect of long term exposure to FIL or whey protein on milk protein gene expression in mammosphere culture. Total RNA was isolated from mammary epithelial cells cultured on EHS for 6 days (see text for details). Results are expressed as arbitrary units of mRNA per 10 μg of RNA.

each protein was observed after treatment with 8 μg/ml whey protein. As FIL is present in the whey fraction, it may be competent to alter gene expression, but its effects should be less than that of the FIL preparation since it is not present in such high concentrations.

In the third experiment experimental conditions were identical to experiment 2, but expression of α_{s1}-casein and β-lactoglobulin after treatment with FIL were investigated. Northern blot analysis indicated that the abundance of α_{s1}-casein and β-lactoglobulin decreased following 72 h incubation with FIL (figure 4.8). Control mRNA abundance was apparently low, however after probing with a 28S cDNA probe, it was became obvious that there were not equal amounts of RNA in each lane (figure 4.8). Densitometric analysis confirmed these observations (table 4.3). When message abundance was corrected for RNA loading, FIL treatment was calculated to have decreased α_{s1}-casein mRNA abundance by 54.2%, while β-lactoglobulin mRNA abundance fell by 52.4%. Treatment with whey also decreased the abundance of milk protein mRNAs in mammosphere culture (figure 4.8).

4.3 DISCUSSION

A cell culture system has been developed for primary culture of goat mammary cells on a reconstituted extracellular matrix derived from the EHS murine tumour. Culture of mammary epithelial cells on this substrata allows formation of polarised three dimensional structures which are morphologically similar to alveoli *in vivo*. These three-dimensional structures, termed mammospheres, synthesise and secrete milk

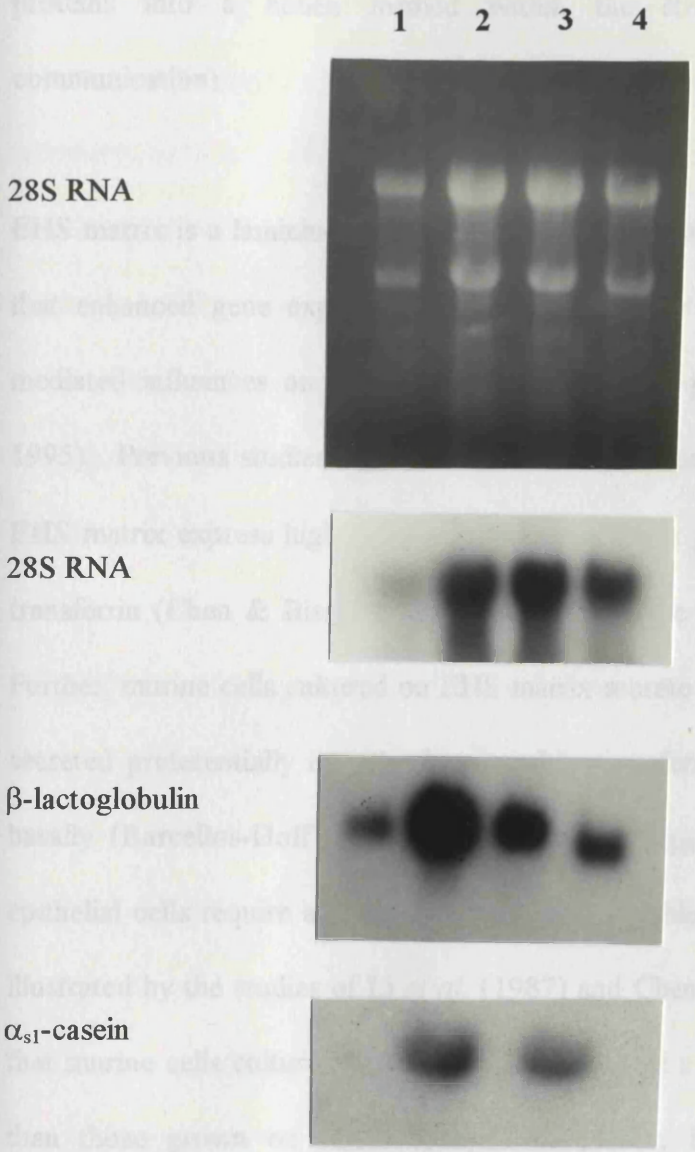


Fig. 4.8 Milk protein mRNA abundance in mammary cells cultured on EHS matrix for 6 days. Total RNA was isolated following 72 h incubation with 8 μg FIL or 8 μg/ml total whey protein, or 4 h incubation with 5 μg/ml brefeldin A. Lane **1**, control; lane **2**, 4h brefeldin A; lane **3**, 72 h whey protein; lane **4**, 72 h FIL.

proteins into a lumen formed within the structure (LMB Finch, personal communication).

EHS matrix is a laminin-rich substratum. A series of experiments have demonstrated that enhanced gene expression on this substratum is the result of laminin-integrin mediated influences on specific milk protein gene promoter regions (Streuli *et al.*, 1995). Previous studies have shown that murine mammary epithelial cells grown on EHS matrix express high levels of the milk protein genes β-casein (Li *et al.*, 1987), transferrin (Chen & Bissell, 1987) and whey acidic protein (Chen & Bissell, 1989). Further, murine cells cultured on EHS matrix secrete proteins vectorially. Caseins are secreted preferentially into the lumen while transferrin is secreted both apically and basally (Barcellos-Hoff *et al.*, 1989; Seely & Aggeler, 1991). Murine mammary epithelial cells require a complex substratum to achieve a fully differentiated state, as illustrated by the studies of Li *et al.* (1987) and Chen & Bissell (1989) which showed that murine cells cultured on EHS matrix exhibited a greater degree of differentiation than those grown on either collagen or plastic. Induction of milk protein gene expression in murine cells cultured on EHS takes place contemporaneously with reorganisation of the cells to form mammospheres (Aggeler *et al.*, 1991).

The current study demonstrates that goat mammary epithelial cells behave similarly to their murine counterparts when cultured on EHS matrix, and express mRNA for the milk protein genes α-lactalbumin, β-lactoglobulin and α_{s1}-casein. In comparison, goat mammary epithelial cells grown on plastic do not express mRNA for these milk protein

genes, suggesting that caprine mammary epithelial cells also require a complex substratum to achieve a fully differentiated state. Formation of mammospheres by goat mammary epithelial cells was accompanied by milk protein gene expression (this study) and synthesis and secretion of milk protein (LMB Finch, personal communication). While the cells reorganised into small multicellular spheres, low levels of gene expression were observed, but by days 5 - 6 of culture when the mammospheres were fully formed and enshrouded in matrix, the cells contained high levels of milk protein mRNA.

Development of this culture system, and confirmation that cells cultured in this way express milk protein genes, has allowed investigation of the factors which direct mammary epithelial cell growth and differentiation, and in particular the role of the feedback inhibitor of lactation (FIL) in the regulation of milk protein synthesis and secretion.

The inhibitory role of FIL has been studied extensively both *in vivo* (Wilde *et al.*, 1988, 1995) and *in vitro* (Wilde *et al.*, 1987, 1989, 1995). Evidence so far suggests that FIL reduces milk yield as a consequence of inhibition of milk secretion, however the exact role of FIL in this scheme is not yet known. It has been shown that FIL acts on an early stage of the secretory process, blocking transport from the ER to the Golgi in a similar way to the drug brefeldin A. Disruption of transport from the ER also results in inhibition of translation, suggesting that accumulation of protein within the ER is able to directly block protein synthesis (Kuznetsov *et al.*, 1992). Such an interpretation

would explain FILs effects on both protein synthesis and secretion. Short-term exposure (4 h) of lactating mouse mammary acini to 8 μg/ml FIL has been shown to inhibit milk protein synthesis and secretion and to disrupt the *trans* Golgi network, an effect also seen with short-term exposure to brefeldin A (Rennison *et al.*, 1993). In the present study, short-term exposure (4 h) of goat mammary epithelial cells on EHS matrix to either 8 μg/ml FIL or 5 μg/ml brefeldin A has no effect on milk protein gene expression. However, 4 h incubation with 5 μg/ml brefeldin A was previously shown to decrease protein synthesis and secretion in mammary acini culture (Rennison *et al.*, 1993). Short-term exposure to brefeldin A also reduced protein synthesis and secretion in goat mammosphere cultures (this study), in a manner similar to that observed in mouse mammary acini culture. The ability of brefeldin A, a known regulator of the secretory pathway (Misumi *et al.*, 1986; Lippincott-Schwartz *et al.*, 1989) to inhibit milk protein secretion without affecting mRNA abundance also suggests that the response is not dependent on up- or down-regulation of milk protein gene expression. This suggests the inhibition of milk protein synthesis and secretion is independent of milk protein gene expression at least in the short-term.

That, in this study, FIL had no effect on milk protein gene expression in the short term, could have been due to the inability of FIL to reach an apically located receptor. Injection of FIL via the teat canal decreases the rate of milk secretion (Wilde *et al.*, 1995). Additionally, auto-immunisation of lactating goats against their own inhibitory protein was found to stimulate milk secretion (Wilde *et al.*, 1996b). An increase in milk secretion was only observed when present in milk but not when present in the

bloodstream (Wilde *et al.*, 1996b). Therefore FIL exerts its effects after it is secreted into milk. For FIL's action on the rate of milk secretion to be an autocrine process, a receptor for FIL must exist on the apical side of the mammary epithelial cell. While such a receptor has not yet been isolated, there is increasing evidence that such a receptor exists.

Experiments in primary culture of mammary epithelial cells suggest that the putative receptor for FIL is found on the apical surface of the luminal epithelial cell. Protein synthesis and secretion in mammary epithelial cells from lactating mice were inhibited by caprine FIL when the cells were cultured in suspension as acini (Rennison *et al.*, 1993). However in mouse mammary epithelial cells cultured as mammospheres FIL had negligible effect on protein synthesis and secretion (Blatchford & Wilde, unpublished observations). Adjacent cells in mammosphere culture form tight intercellular junctions such that proteins in culture medium are excluded from the lumen of the mammosphere. The apparent insensitivity of mammosphere cultures indicates lack of access to an apical receptor. When FIL was allowed access to the luminal space by transiently opening intercellular tight junctions, protein secretion was inhibited as in acini cultures (Wilde *et al.*, 1996a; Wilde & Peaker, 1996). In the present study mammosphere integrity (estimated by the percentage luminal secretion) was less than that of intact murine mammospheres but greater than that of those with leaky intercellular junctions, suggesting that FIL should have had some access to its receptor (Wilde & Blatchford, unpublished observations). Therefore, lack of effect on

milk protein mRNA in the short term suggests that this is not required for inhibition of protein secretion.

Inhibition of protein synthesis and secretion after longer exposure of mammosphere cultures to FIL indicates that, with time, FIL did indeed gain access to its site of action on the apical cell membrane. As already indicated, this may have been because mammospheres were incompletely formed: the proportion of protein secreted apically was lower than in well-formed mouse mammosphere cultures (DR Blatchford, personal communication). Also, with time FIL may reach the lumen of the mammosphere by transcytosis, or by a paracellular route: studies in mouse mammospheres suggest that there is transient opening of intercellular junctions and redistribution of proteins between lumina and culture medium over extended periods (Hurley et al., 1994). Whatever its route of access, this series of experiments demonstrate that long term exposure of goat mammospheres to FIL inhibits milk protein gene expression.

The mechanism behind these effects is unknown. However, the regulation of secretory cell differentiation by FIL may involve local modulation of the secretory cells' sensitivity to circulating hormones (Wilde *et al.*, 1990). Down-regulation of prolactin receptors in rodents inhibits milk protein gene expression (Eisenstein & Rosen, 1988), and expression of the major milk protein genes is regulated by prolactin at the level of transcription and mRNA stability (Guyette *et al.*, 1979). The role of prolactin is less clear in ruminants, although prolactin depletion in lactating goats does reduce milk yield (Knight *et al.*, 1990). However, FIL's modulation of mammary hormone

receptors is unlikely to be limited to prolactin - autocrine feedback may well down-regulate receptors for other galactopoietic hormones and so down-regulate milk protein gene expression. Implicit in this scheme is that FIL may regulate its own expression and secretion. Elucidation of these interdependent events requires that the gene for FIL be identified and characterised. Chapters five and six therefore centre on attempts to clone FIL.

Chapter Five

Molecular cloning of the FIL gene by immunoscreening

5.1 INTRODUCTION

Milk secretion is under the control of a locally acting feedback inhibitor of lactation (FIL). The way in which FIL regulates milk synthesis and secretion is as yet unknown, but evidence so far suggests that it reduces milk yield as a direct consequence of inhibition of the secretory pathway (Rennison *et al.*, 1993). Inhibition of the secretory pathway in turn inhibits synthesis of milk constituents. As a probable consequence of its effect on membrane trafficking, FIL may also have longer term effects on hormone receptor distribution and number (McKinnon *et al.*, 1998; Bennett *et al.*, 1990). Therefore, FIL is also a potential regulator of epithelial cell differentiation within the tissue. This is consistent with the sequential effects of milk removal on mammary function (Wilde *et al.*, 1987b). Changes in the frequency of milk removal regulates milk secretion acutely and this altered rate of milk secretion is sustained by changes in cell differentiation and, ultimately, modulation of secretory cell number (Knight *et al.*, 1990; Wilde *et al.*, 1990). We wished to investigate whether FIL's milk concentration is regulated in a manner consistent with involvement in these sequential responses *in vivo*. Cloning the gene for FIL is crucial to the understanding of the control of FIL's milk concentration and the regulation of FIL gene expression by FIL itself.

Study of eukaryotic gene structure and expression relies on the availability of cloned genes as probes. Isolation of complementary DNA (cDNA) clones for milk protein genes allows analysis of developmental or tissue-specific gene expression, while isolation of genomic clones may reveal important non-transcribed regions which are

not represented by cDNA clones. In addition, sequence analysis may identify regions of homology and possible regulatory elements common to other milk protein genes. Advances in molecular biology have made possible the identification of genomic and cDNA clones for a variety of milk protein genes, thereby providing the tools for detailed study of mammary gene expression and its regulation (reviewed by Mercier & Vilotte, 1993; Groenen & van der Poel, 1994).

DNA cloning has four essential stages: generation of DNA fragments; ligation of the DNA fragments into a vector; introduction of the vector into a host cell; selection of clones of interest.

The choice of library or screening method depends very much on the nature of the gene to be cloned. Isolation of eukaryotic genes usually involves construction of a cDNA library which represents the mRNA population of a particular tissue or cell type. The abundance of a specific cDNA clone within a library is proportional to the abundance of that particular mRNA within the entire mRNA population. Isolation of cDNA clones for rare mRNAs necessitates the construction of cDNA libraries containing 10^5 to 10^7 recombinants. The high efficiency and reproducibility of *in vitro* packaging of λ vectors is ideal for obtaining large numbers of cDNA clones. The two λ vectors most commonly used for library construction are λgt10 and λgt11 (reviewed by Huynh *et al.*, 1988).

Cloned sequences of interest can be selected from recombinant DNA libraries by screening the library with synthetic oligonucleotide probes, antibody probes or cDNA probes representing differentially expressed genes. Libraries constructed in λgt10 may be screened with nucleic acid probes, while those constructed in λgt11 may be screened with either nucleic acid or antibody probes. FIL has not been cloned in any other species and the protein had proved difficult to sequence when cloning was first attempted. However it was possible to purify FIL in amounts sufficient to produce anti-FIL antibodies. Consequently, initial attempts to clone FIL were based on immunological screening of a goat cDNA library constructed in the bacteriophage vector λgt11.

This chapter describes the screening of a lactating goat mammary cDNA library with an antibody raised against the bovine Feedback Inhibitor of Lactation (FIL), with the aim of identifying cDNA encoding goat FIL.

5.2 RESULTS

5.2.1 Suitability of α-bovine FIL antibody for immunoscreening

The ideal antibody for use in immunological screening should be polyclonal, have absolute specificity for the protein of interest, have a high titre and belong to the IgG class of immunoglobulins (Maniatis *et al.*, 1989).

Antibodies raised to caprine FIL were found to cross-react with other goat whey proteins (Wilde *et al.*, 1995). Therefore the antiserum used in the present study was

raised against the bovine feedback inhibitor of lactation, a protein in cow's milk which has similar inhibitory activity to caprine FIL. The bovine protein, like its caprine counterpart, was identified by its ability to inhibit reversibly both casein and lactose synthesis in lactating rabbit mammary explants (Addey *et al.*, 1991b). Bovine FIL was purified by anion exchange chromatography from a M_r 6,000 - 30,000 fraction of bovine whey proteins as described by Addey *et al.* (1991b), and was collected as the second resolved fraction (figure 5.1). A polyclonal antiserum was produced as described in section 2.5.1.

As mentioned previously, antibodies for immunoscreening should have absolute specificity for the protein of interest. The specificity of the antiserum used in this study was determined by Western blotting. Caprine FIL was purified by anion-exchange chromatography from a M_r 6,000 - 30,000 fraction of caprine whey proteins (section 2.3.2), yielding a single whey protein of M_r 7,600. Under reducing conditions on SDS-PAGE this protein migrates with an apparent M_r of 66,000 (figure 5.2) a phenomenon attributable to its oligosaccharide content (Wilde *et al.*, 1995). Western blotting with the α-bovine FIL antibody detected a single protein band with apparent M_r 66,000 in immunoblots of unfractionated whey (figure 5.2).

Another important consideration is whether the antibody recognises the deglycosylated form of the protein. Proteins expressed in *E. Coli* will not be glycosylated. Wilde *et al.* (1995) showed that deglycosylated FIL migrated on SDS-PAGE with an M_r of 7,000 (an apparent M_r consistent with its elution during gel filtration) and that the

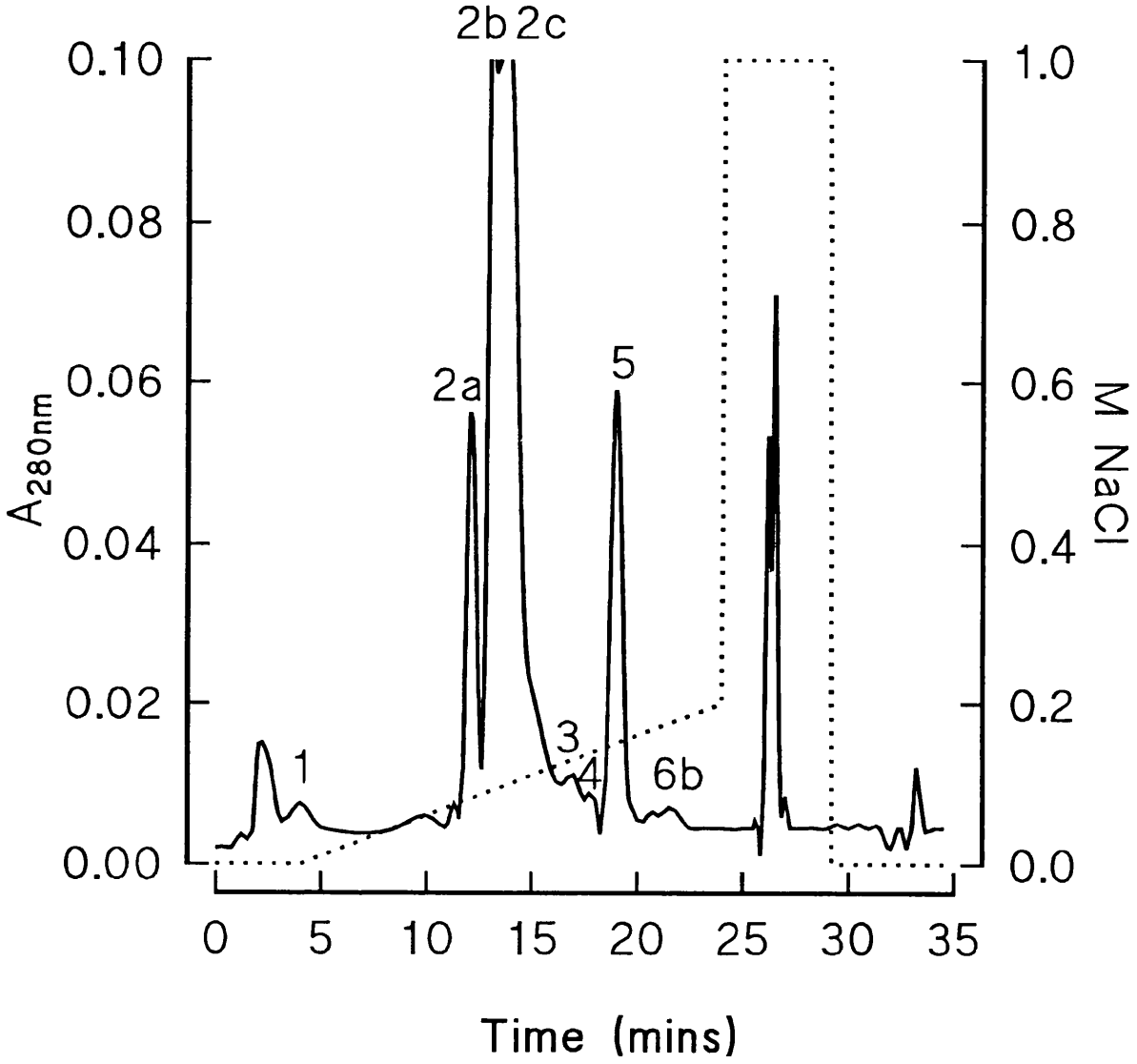


Figure 5.1 Resolution of a M_r 6,000 - 30,000 bovine whey fraction. The bovine whey fraction was resolved by anion exchange chromatography, using a Mono Q HR 10/10 column (FPLC System, Pharmacia), 10 mM imidazole, pH 7.0 and a 0-1.0 M sodium chloride gradient. Bovine FIL was collected as the second resolved fraction (2).

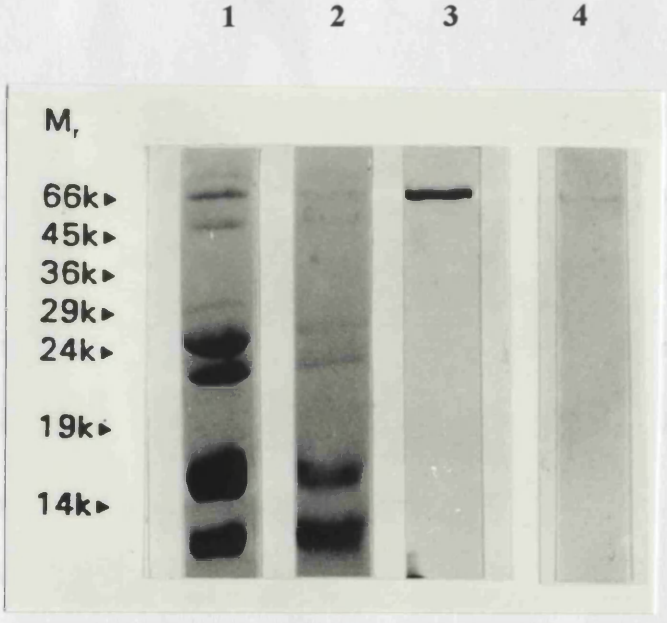


Figure 5.2 Detection of FIL using a specific polyclonal antiserum. Antiserum was raised in the rabbit against a protein with similar inhibitory activity to goat FIL purified from bovine milk. Lane 1, SDS PAGE of caprine whey proteins; lane 2, SDS PAGE of a M_r 6,000 - 30,000 fraction of caprine whey proteins; lane 3, anion-exchange purified caprine FIL; lane 4, immunoblot detection of inhibitory protein in a M_r 6,000 - 30,000 fraction of caprine whey proteins.

antibody recognised a single protein species of this size. From these observations it was concluded that the antibody is specific for the polypeptide and not the oligosaccharide moiety of the protein.

Preparations of IgG generally give lower backgrounds than whole sera, therefore the IgG fraction of the α-bovine FIL antiserum was isolated by ammonium sulphate precipitation and purified by affinity chromatography on Protein A agarose (section 2.5.2). Antibodies recognising coliform proteins are found in many polyclonal antisera preparations, and these were removed from the α-bovine FIL antibody prior to screening by immunoabsorption of the antibody with an *E. coli* extract made by freeze thawing and sonication (sections 2.6.2 & 2.6.3).

It is important that the antibody recognises FIL when the protein is spotted onto nitrocellulose since the recognition of protein in this form is the basic principle of immunological screening. The α-bovine FIL antibody was capable of recognising as little as 200 pg of both native and denatured FIL in an area equivalent to that occupied by a single bacterial plaque (figure 5.3).

5.2.2 Immunoscreening

α-FIL IgG was used to screen a random primed cDNA library prepared from lactating goat mammary poly (A) RNA in λgt11 (section 2.6.5). Immunoscreening was carried out as described in section 2.6.6. In libraries constructed in λgt11 the cloned sequence is fused to the carboxy terminus of the β-galactosidase gene of λgt11. Fusion of the

A

B

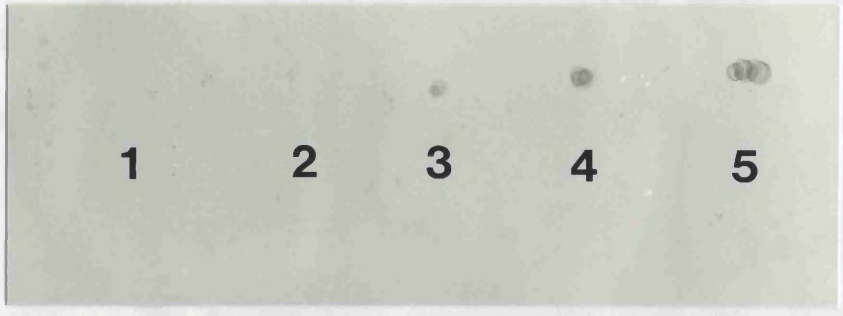


Figure 5.3 Antibody recognition of FIL on nitrocellulose. Varying amounts of anion-exchange purified caprine FIL were spotted onto Immobilon P nylon membrane in an area equivalent to that occupied by a bacterial plaque. FIL was detected by immunoblotting using a specific polyclonal antiserum. **A** - native protein, **B** - denatured protein. Lane **1**, 50 pg; lane **2**, 100 pg; lane **3**, 200 pg; lane **4**, 500 pg; lane **5**, 1 ng FIL.

cloned sequence to the β-galactosidase gene both stabilises and ensures high expression of the fusion protein.

The library was grown on a lawn of *E. coli* Y1090 cells. Once a large number of infected cells surrounded each plaque (after 3-4 h), *lacZ* directed gene expression was induced by placing a nitrocellulose filter soaked with isopropyl-1-thio-β-D-galactoside over the plate. Protein released from the lytically infected cells was immobilised on the nitrocellulose filters, and bound antibodies were detected by incubation with α-FIL IgG, followed by incubation with an appropriate anti-IgG conjugated to alkaline phosphatase. Following addition of the alkaline phosphatase substrate plaques which expressed a fusion protein recognised by α-FIL IgG were identified as a purple colour on the nitrocellulose filter.

In an initial screen of 10^5 recombinants, 11 immunoreactive clones were identified, six of which remained immunoreactive in subsequent plaque purifications. Phage DNA was isolated from these immunoreactive clones as described in sections 2.6.8 - 2.6.9. Restriction enzyme digestion of the phage DNA was carried out to establish the presence of inserted DNA. The enzyme *ECo*R I recognises the *ECo*R I site within the synthetic adaptors used to ligate the cDNA and the λ gt11 vector, allowing inserted DNA to be separated from phage DNA. Digestion of the phage DNA with *ECo*R I confirmed that each of the 6 immunoreactive clones contained foreign DNA (figure 5.4). Two of these clones, J1(3A) and J2(8B), were selected and the phage DNA was analysed.

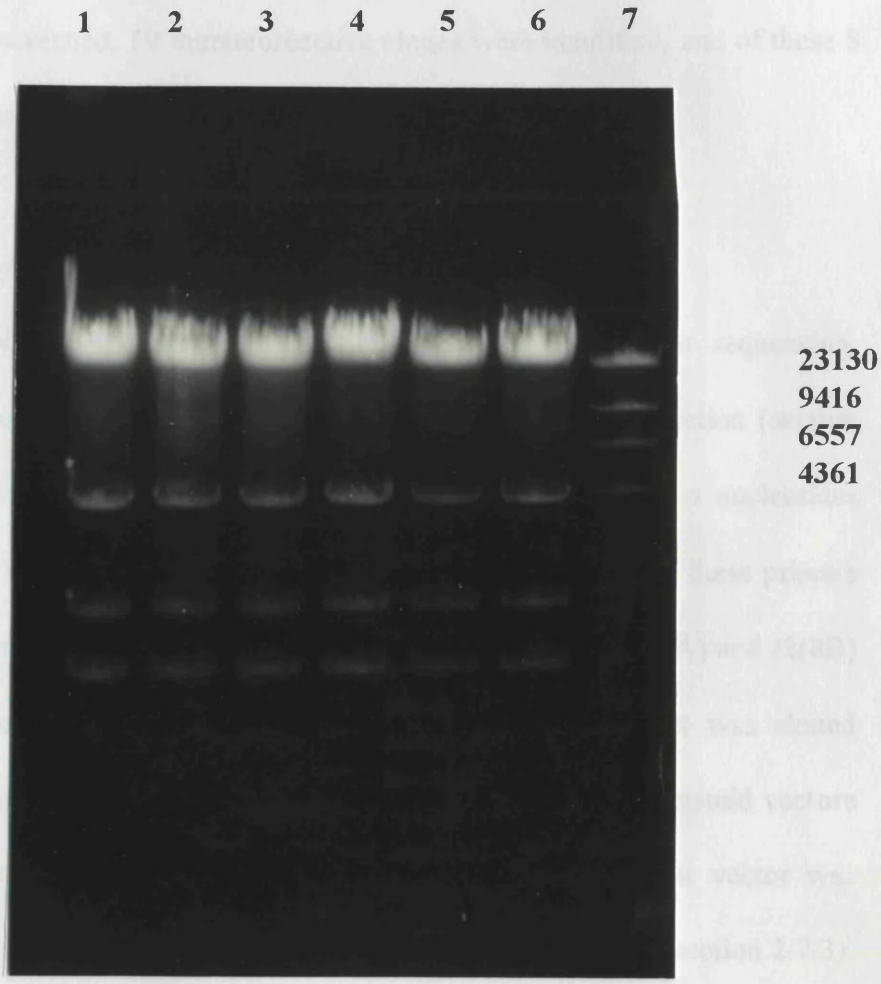


Figure 5.4 Restriction enzyme digest of phage DNA. Digestion with the restriction enzyme *ECo*R I confirmed the presence of inserted DNA. Lanes **1-6**, *ECo*R I digested DNA from 6 phage lysates; lane **7**, λ DNA digested with *Hin*d III.

During analysis of the immunoreactive clones from the first immunoscreening experiment a further screening experiment was carried out. Again a total of 10^5 recombinants were screened, 19 immunoreactive clones were identified, and of these 8 remained immunoreactive in subsequent plaque purifications.

5.2.3 Preparation of DNA for sequencing

The amount of DNA released from phage lysates was insufficient for sequencing. Therefore, the cloned DNA was amplified by the polymerase chain reaction (section 2.7.1) using the primers BG1 and BG2 which were complementary to nucleotides 2969-2998 and 3035-3064 of the β-galactosidase gene of λgt11. Use of these primers allowed amplification of the cloned DNA sequence only. Amplified J1(3A) and J2(8B) are shown in figure 5.5. Following amplification, each PCR product was cloned directly into the Xcm I site of the modified pGEM 5zf (+) series of phagemid vectors using T4 DNA ligase, as described in section 2.7.2. The recombinant vector was introduced into *E. coli* strain JM109 by transformation of the host cells (section 2.7.3). Cells were transformed successfully with the vector containing J1(3A), however transformation with J2(8B) was unsuccessful. Digestion of plasmid DNA with *Nco* I allowed analysis of the foreign DNA. In the modified pGEM 5zf (+) vector utilised, a *Nco* I site flanks each end of DNA inserted at the Xcm I site. Plasmid DNA was prepared (section 2.7.4) from six randomly chosen transformed colonies, and as shown in figure 5.6, five clones contained a single insert, while one clone (lane 5), which did not, was discarded. The inserts in the remaining 5 clones appeared to be of a similar

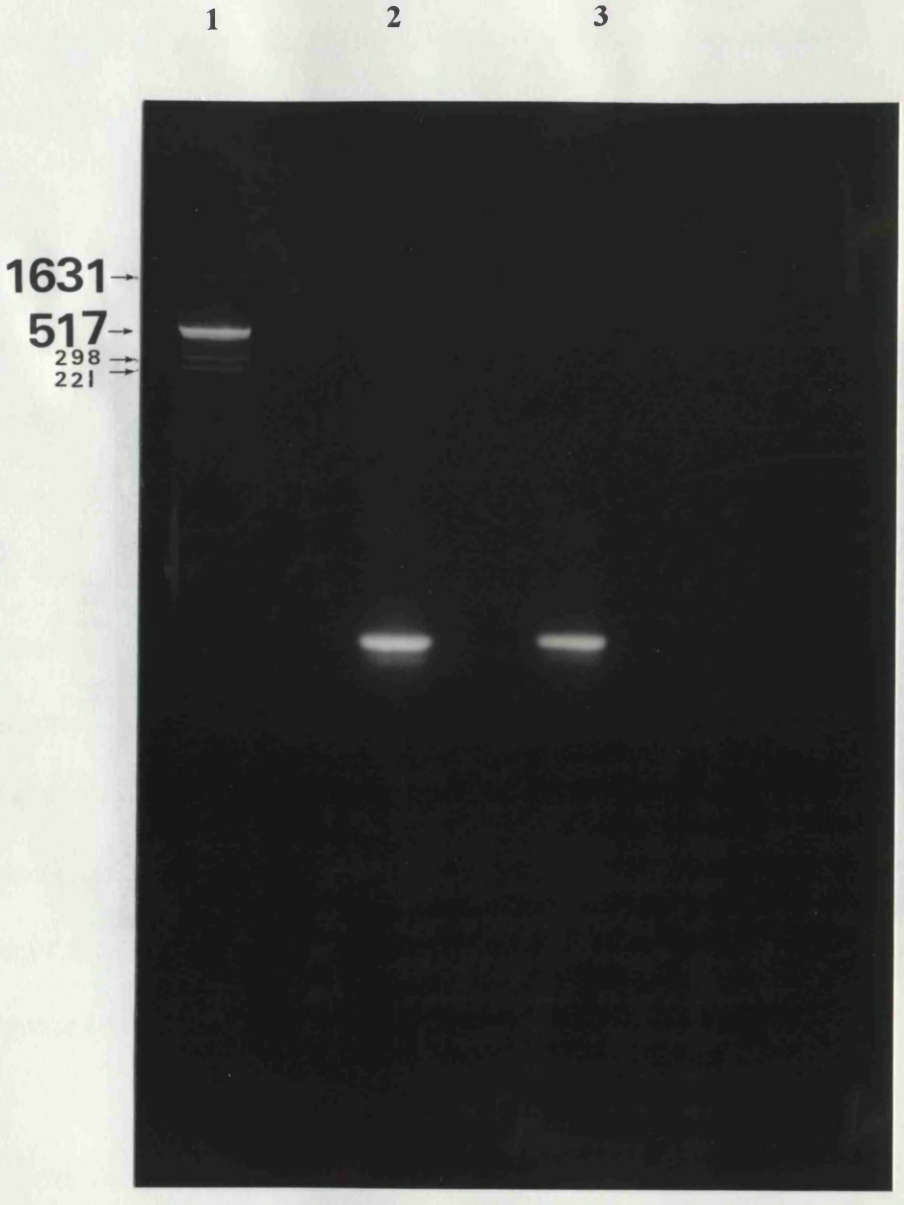


Figure 5.5 Amplification of DNA using the polymerase chain reaction. Lane **1**, pAT 153 digested with *Hin*d III; lane **2**, amplified J1(3A); lane **3**, amplified J2(8B).

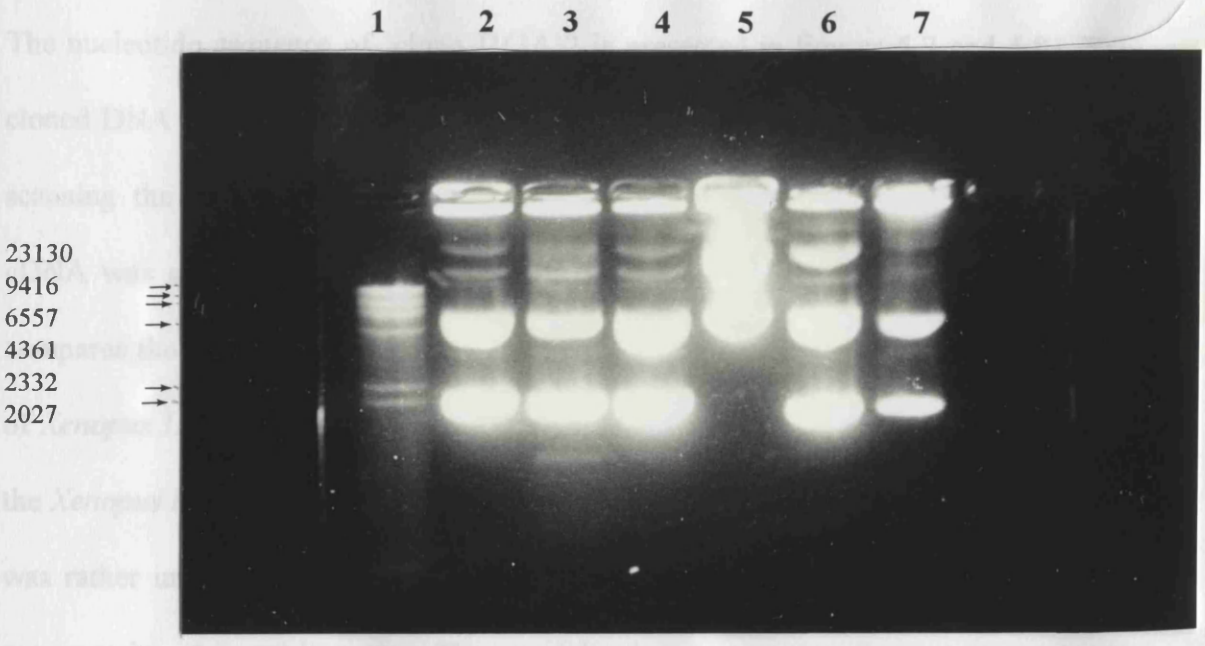


Figure 5.6 Restriction enzyme digest of plasmid DNA. Plasmid DNA prepared from clone J1(3A)2 was digested with the restriction enzyme *Nco* I to confirm the presence of inserted DNA. Lane **1**, λ DNA digested with *Hin*d III; lanes **2-7**, plasmid DNA digested with *Nco* I.

size, and one of these clones, designated J1(3A)2 was sequenced as described in sections 2.7.6 - 2.7.7.

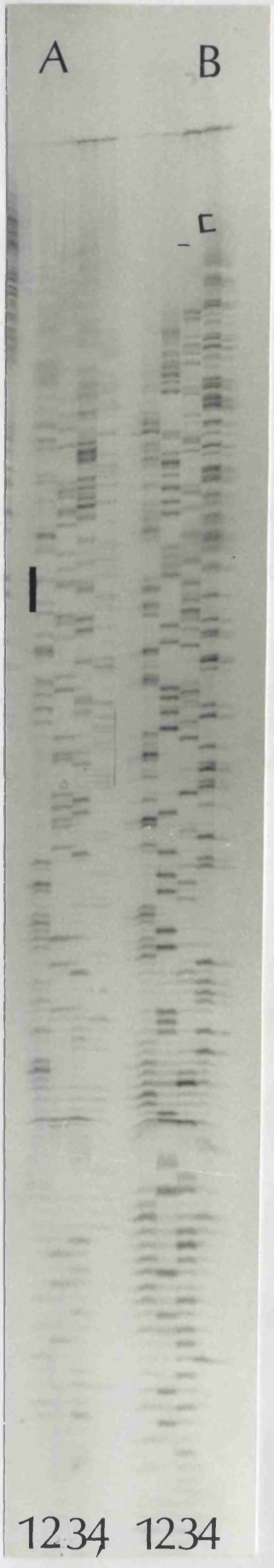
5.2.4 Sequence analysis

The nucleotide sequence of clone J1(3A)2 is presented in figures 5.7 and 5.8. The cloned DNA consisted of 163 base pairs. The sequence information was analysed by scanning the EMBL database using FASTA, and analysis revealed the sequenced cDNA was similar to ribosomal RNA genes from a variety of species. Figure 5.9 compares the nucleotide sequence of the J1(3A)2 clone with the nucleotide sequence of *Xenopus Laevis* 28S ribosomal RNA gene. J1(3A)2 exhibited 81.6% homology to the *Xenopus Laevis* 28S ribosomal RNA gene in 163 base pairs of overlap. This result was rather unexpected, as the antibody appeared to be recognising a protein whose sequence is not found in nature. The remaining immunoreactive clones were analysed as described below (section 5.2.5).

5.2.5 Analysis of other possible positives

As described above, the identity of the sequenced DNA was rather unexpected. In order to discover whether all clones identified by the antibody were similar to the sequenced clone, a cDNA probe was prepared from the sequenced DNA (section 2.7.9). Following digestion with the restriction enzyme *Nco* I, DNA was electrophoresed on a low melting point agarose gel and the band corresponding to the cloned DNA was excised (figure 5.10). The cDNA probe was then labelled with [α-^{32}P]dCTP (section 2.2.5). The immunoreactive clones identified by immunoscreening

Figure 5.7 Dideoxy chain termination sequencing of DNA. A - forward reaction, B - reverse reaction. Lane 1, C; lane 2, T; lane 3, A; lane 4, G.


A
B
1234 1234

5' GGGTGGGTCTTCCCGTACCGGCACATGTCCGCGGACCAACCGCGGGGCGGGGATT

3' CCCACCCAGAAGGGCATGGCCGTGTACAGGCGCCTGGTTGGCGCCCCGCCCCTAA

CAGGACGCTGGGCTCTTCCTGTTCACTCGCGTTACTGAGGGAATACCTGGTTAGT

GTCCTGCGACCCGAGAAGGACAAGTGAGCGCAATGACTCCCTTATGGACCAATCA

TTCTTTTCCTCCGCTGACTTAATATGCTTAAATTCAGCGGGTCGCGCCTCACC **3'**

AAGAAAAGGAGGCGACTGAATTATACGAATTTAAGTCGCCCAGCGCGGAGTGG **5'**

Figure 5.8 Nucleotide sequence of clone identified by immunoscreening

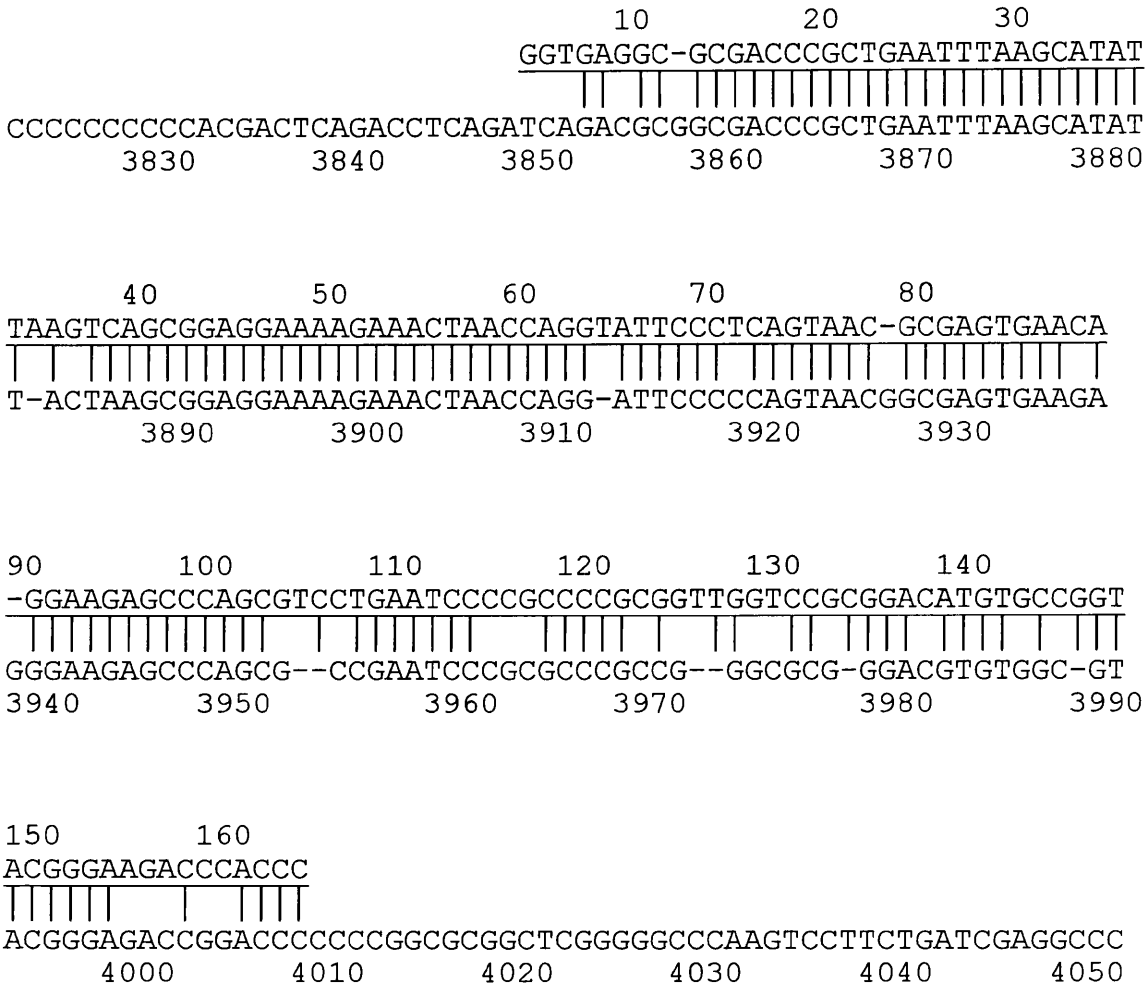


Figure 5.9 Nucleotide sequence comparison. Comparison of the nucleotide sequence of clone J1(3A)2 with the nucleotide sequence of *Xenopus laevis* 28S RNA, showing region of highest homology. The underlined sequence corresponds to that of clone J1(3A)2.

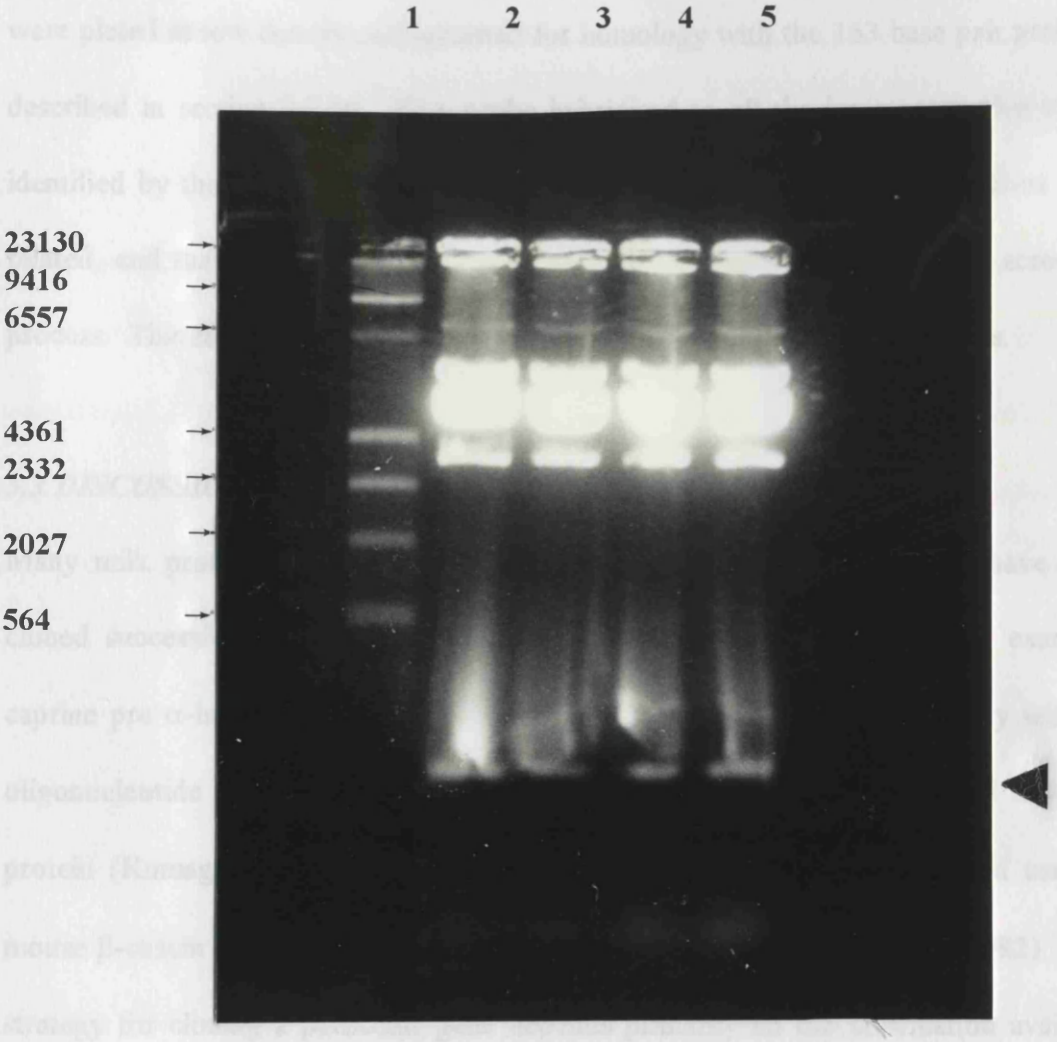


Figure 5.10 cDNA probe preparation. DNA was digested with the restriction enzyme *Nco* I and resolved on a low melting point gel. Lane 1, λ DNA digested with *Hin*d III, lanes 2-5, *Nco* I digested DNA. The bands marked ➔ were excised and used as a cDNA probe.

were plated at low density and screened for homology with the 163 base pair probe as described in section 2.7.10. This probe hybridised to all the immunoreactive clones identified by the antibody. These results suggest that all the possible positives were related, and that the antibody recognised the same epitope throughout the screening process. Therefore the sequence described in section 5.2.4 was not erroneous.

5.3 DISCUSSION

Many milk proteins, including a number of caprine milk protein genes, have been cloned successfully (for review see Groenen & van der Poel, 1994). For example, caprine pre α-lactalbumin cDNA was isolated by screening a cDNA library with an oligonucleotide probe based on the amino acid sequence of a portion of the goat protein (Kumagai *et al.*, 1987), and the caprine β-casein gene was cloned using a mouse β-casein cDNA probe to screen a genomic library (Roberts *et al.*, 1992). The strategy for cloning a particular gene depends primarily on the information available about the gene or its gene product.

The feedback inhibitor of lactation has been identified in the milk of goats (Wilde *et al.*. 1995) and cows (Addey *et al.*, 1991b); and a protein fraction with similar activity has been identified in both humans (Prentice *et al.*, 1989) and in a macropod marsupial (Hendry *et al.*, 1992). However, the gene for FIL has not been cloned from any species, therefore it was not possible to isolate the caprine gene using a cDNA probe derived from a previously cloned FIL gene. Further, little protein sequence was available for FIL when this study began, therefore library screening with synthetic

oligonucleotides constructed on the basis of protein sequence was not initially an option.

Consequently, attempts to clone the gene for FIL centred on immunological screening. While protein sequence information was limited, it was possible to purify both the caprine and bovine proteins (Addey *et al.*, 1991b; Wilde *et al.*, 1995), and this led to the prospect of antibody production. Antisera raised to caprine FIL cross reacted with other caprine whey proteins, however, the antiserum raised to bovine FIL was found to be specific for the caprine protein (Wilde *et al.*, 1995 & this study).

Several genes have been successfully cloned using antibodies to screen both genomic and cDNA libraries. The first reported results were those of Young and Davis (1983b) who isolated yeast RNA polymerase II subunit encoding genes using a polyclonal antibody directed against purified yeast RNA polymerase II. Other examples include the cloning of firefly luciferase (de Wet *et al.*, 1985), brain glutamate decarboxylase (Kaufman *et al.*, 1986) and thyrotropin-releasing hormone precursor (Lechan *et al.*, 1986).

Clones J1(3A) and J2(8B) showed the strongest signals with the antibody. Subsequent sequencing of one of these clones, J1(3A), and DNA sequence analysis revealed striking homology to ribosomal RNA genes from a variety of species. Clearly this result was unexpected, but further investigation revealed that all the clones identified in this study were related to the same sequence. Clone J2(8B) was not sequenced as the

vector containing this clone was not successfully introduced into an *E. Coli* host. Transformation is an extremely inefficient process, no matter how carefully the process is carried out, and generally only 0.01% of the plasmid molecules are transported into the host cell. That *E. Coli* was not transformed with J2(8B) was not unusual, and, in any case when this recombinant was screened with a cDNA probe derived from clone J1, both these clones were found to be related.

Subsequent screening of all other positive clones identified by immunological screening revealed that all were false positives. Similar problems have been encountered by others using this method. Boutin *et al.* (1988) experienced problems when using antibodies to isolate cDNA for the rat prolactin receptor. All clones identified by both monoclonal and polyclonal antibodies against the rat prolactin receptor were found to be false positives, despite the apparent specificity of their antiserum for the prolactin receptor. No explanation was offered for this result. Cowell & Hurst (1992) reported difficulty using immunoscreening to clone several transcription factors. In this case, failure of immunoscreening was ascribed to the presence of a minor but extremely antigenic contaminant in highly purified transcription factor preparations. This minor contaminant generated a strong immunogenic response even when present in undetectable amounts. Clearly, this confirmed the intuitive prediction that antibody specificity is a key factor in the success, or otherwise, of any immunoscreening approach.

It is conceivable that processing of FIL for antiserum production, or the nature of the immunoscreening process may have contributed to the detection of false positives, and conversely, the failure to detect true positive clones encoding FIL sequences. Although experiments have indicated that FIL is still recognised by the antibody after deglycosylation (Wilde *et al.*, 1995), an absence of carbohydrate moieties on the recombinant protein may mean that many epitopes present may not be recognised by the polyclonal antiserum. Also, FIL is a small protein of M_r 7,600, and as such may have only a small number of antigenic determinants. Even minor contamination with a larger, more antigenic protein may produce a more complex and potentially higher affinity immune response. It is also possible that bovine albumin to which FIL was conjugated may contain a minor antigenic contaminant. However, it should be emphasised that immunoblotting confirmed the specificity of the antibody for its intended target before library screening was attempted, indicating that cDNA encoding FIL, if expressed in λgt11, should have been identified by this approach.

The library used in this study was a random primed goat mammary cDNA library constructed in λgt11. Use of random primed libraries is advantageous when the message size of a protein is unknown. While FIL has been shown to be a small protein of M_r 7,600 it does not necessarily follow that the gene for FIL will also be small. First strand synthesis is primed with random oligonucleotide primers which hybridise at random sites along the mRNA template. Random priming ensures equal representation of all sequences in the mRNA population, and that all regions of a protein encoded by a particular mRNA are expressed in the library. However, random primed cDNA

libraries may contain 75% ribosomal sequences since ribosomal RNA can account for as much as three quarters of the total RNA within the cell. This problem may be circumvented by priming first strand synthesis with an oligo dT primer. This primer hybridises to the 3' terminal poly (A) sequence of the mRNA template and cDNA synthesis proceeds from a determined site on the template rather than at random sites throughout it. Therefore, oligo dT primed cDNA libraries contain no ribosomal sequences.

The quality of the cDNA library is also important when considering immunoscreening. The library used in this series of experiments had an average insert size of 300 base pairs, due at least in part, to the inefficiency of the size separation stage during library preparation. Consequently, only small fusion proteins were expressed, and it is possible that the antigenic portion of FIL was not represented, allowing identification of an antigenically similar but unrelated protein expressed in the library.

In conclusion, this chapter has demonstrated some of the problems associated with immunoscreening, and that it is unwise to rely on a single method for identifying a gene. Since the α-FIL antibody appears to recognise a ribosomal sequence expressed in the random primed library, it was decided to construct and screen an oligo dT primed goat mammary cDNA library in a further attempt to isolate cDNA for caprine FIL. Screening such a library would circumvent the problems experienced with immunoscreening.

Chapter Six

Further strategies to clone FIL

6.1 INTRODUCTION

As discussed in chapter five, cloning the gene for the feedback inhibitor of lactation (FIL) is crucial to the understanding of the control of FIL's milk concentration and the regulation of FIL gene expression. As the immunoscreening method was unsuccessful, this chapter describes further strategies which were implemented in an attempt to clone the gene for FIL.

Three strategies were attempted concurrently. As one approach, a new goat mammary cDNA library was constructed. Secondly, oligonucleotides were designed for use in library screening. Thirdly, a polymerase chain reaction-based procedure was also attempted in order to identify cDNA for FIL.

Library construction

Library screening using an antibody specific for FIL was unsuccessful as the antibody recognised a ribosomal sequence expressed in the random primed cDNA library used in this procedure. This problem may be circumvented by priming first strand synthesis with an oligo dT primer. This primer hybridises to the 3' terminal poly (A) sequence of the mRNA template, and cDNA synthesis proceeds from a determined site on the template rather than at random sites throughout it. Therefore oligo dT primed libraries contain no ribosomal sequences.

Design of oligonucleotide probes

In addition to the identification of genes using antibodies raised to their gene products, several other approaches may be used. Genes of interest may be identified not only by antibody probes but also by the use of synthetic oligonucleotide probes or cDNA probes representing differentially expressed genes. Antibody screening has so far proved unsuccessful, and, as the gene for FIL has not yet been cloned in any other species, a cDNA probe for FIL was not available. The use of synthetic oligonucleotide probes, designed on the basis of known protein sequence has achieved identification of several genes for proteins whose partial amino acid sequence is known. Genes cloned in this way include bovine trypsin inhibitor (Anderson & Kingston, 1983) and caprine pre α-lactalbumin (Kumagai *et al.*, 1987).

There are three types of oligonucleotide probes commonly used - single oligonucleotides of defined sequence, pools of short highly degenerate oligonucleotides, and longer oligonucleotides of lesser degeneracy. The use of longer, less degenerate oligonucleotides, or guessmers, is the method of choice when sufficient protein sequence - usually 10 or more amino acids - is available for their design. Guessmers are synthetic oligonucleotides which range in size from 30 -70 nucleotides and contain only a subset of all possible codons at each position. Most amino acids are coded for by codons which differ only in the third nucleotide position, therefore at least two of the three nucleotides are guaranteed to match their target perfectly. The detrimental effect of any mismatch is outweighed by the increased stability of hybrids formed by longer oligonucleotides (Binnie, 1991). Lathe (1985) calculated that a

probe would have 76% homology to its target sequence even if all codon choices were made on a random basis. It follows, therefore, that if codon utilisation in the species of interest is taken into consideration, the homology of the probe will increase further, making it more specific.

When cloning was first attempted, little sequence data was available, and so only immunoscreening was possible. However, further protein sequencing attempts yielded a consensus sequence for the N-terminal portion of the FIL protein (Wilde et al., 1995; see figure 6.1). Searching of Swissprot and OWL databases with this amino acid sequence revealed no homology with other milk proteins or with any other known protein. Knowledge of this partial protein sequence allowed construction of guessmers for use in a variety of protocols aimed at cloning the gene for caprine FIL.

PCR Screening

Synthetic oligonucleotides constructed from the FIL consensus sequence were also used in the RACE protocol. RACE, or rapid amplification of cDNA ends, is a polymerase chain reaction-based technique which facilitates the cloning of full length 5' and 3' cDNAs after a partial amino acid sequence has been obtained. It is a technique which lends itself to screening when only N-terminal sequence is available; the first primer can be designed from the partial N-terminal sequence, while the second primer can be an oligo dT sequence binding the poly (A) region of the mRNA of interest.

Ala-Pro-Pro-Phe-Glu-Arg-Asn-Ser-Pro-Gly-Arg-Leu-

Figure 6.1 Partial amino acid sequence of FIL. N-terminal analysis of the inhibitory protein produced a consensus sequence consisting of 12 amino acids. Searching of Swissprot and OWL databases revealed no homology with any known protein.

6.2 RESULTS

6.2.1 Designing the optimal oligonucleotide

There are two crucial considerations when designing synthetic oligonucleotide probes (reviewed by Binnie, 1991). The sequence of each oligonucleotide must be designed in order that it has the highest chance of binding its target sequence, and the temperature and conditions in which hybridisations are performed must be calculated to maximise hybridisation specificity. Hybridisation conditions are discussed in detail in section 6.2.5.

6.2.2 Codon usage in the goat

Codon usage in the goat was investigated using the coding sequences of five caprine milk protein genes, β-casein (Roberts *et al.*, 1992), α_{s2}-casein (Bouniol, 1993), pre α-lactalbumin (Kumagai *et al.*, 1987), κ-casein (Coll *et al.*, 1993) and β-lactoglobulin (Folch *et al.*, 1993). Frequency of use of each possible codon for each amino acid is represented in table 6.1, with the frequency of use being expressed as a percentage of the total appearance of that amino acid.

6.2.3 Codon choice

The sequences of synthetic oligonucleotides JMB 1 - 4 are shown in figure 6.2 JMB 1 and 3 are oriented in the sense direction, while JMB 2 and 4 are antisense versions of JMB 1 and 3, respectively. Two distinct oligonucleotides were synthesised in each orientation to minimise the impact of serine (position 8) which displayed no particular codon bias. Sense oriented oligonucleotides were constructed for use in 3' RACE

Amino Acid	Codon	α-lac	β-cas	α_{s2}-cas	κ-cas	β-lac
Ala (A)	GCT	14	12.5	36	16.5	15
	GCC	57	50	45	39	65
	GCA	29	37.5	19	39	5
	GCG	0	0	0	5.5	15
Arg (R)	CGT	0	0	0	20	0
	CGC	0	0	14	0	0
	CGA	0	0	0	0	0
	CGG	0	0	0	0	33
	AGA	0	100	57	40	33
	AGG	100	0	29	40	33
Asn (N)	AAT	43	50	73	57	0
	AAC	57	50	27	43	100
Glu (E)	GAA	50	53	63	55	0
	GAG	50	47	37	45	100
Gly (G)	GGT	33	40	50	50	0
	GGC	17	0	50	0	83
	GGA	50	40	0	50	0
	GGG	0	20	0	0	17
Leu (L)	TTA	6	0	0	15	0
	TTG	23	0	12.5	15	7.5
	CTT	6	35	0	7.5	7.5
	CTC	23	19	37.5	7.5	18
	CTA	6	4	12.5	15	0
	CTG	36	42	37.5	38	67
Phe (F)	TTT	33	43	44	57	0
	TTC	67	57	56	43	100
Pro (P)	CCT	100	53	15	27	12.5
	CCC	0	31	46	0	62.5
	CCA	0	12.5	31	68	12.5
	CCG	0	3.5	8	5	12.5
Ser (S)	TCT	25	33	21.5	8	0
	TCC	25	13	28.5	31	33
	TCA	12.5	7	21.5	15	0
	TCG	0	0	0	0	0
	AGT	25	14	21.5	46	33
	AGC	12.5	33	7	0	33

Table 6.1 Codon usage in the goat. The relative frequencies of use of each codon are expressed as a percentage of all the codons coding for a particular amino acid.

JMB 1

5' GCC CCT CCT TTT GAG AGA AAC AGT CCT GGT 3'

JMB 2

3' CGG GGA GGA AAA CTC TCT TTG TCA GGA CCA 5'

JMB 3

5' GCC CCT CCT TTT GAG AGA AAC TCC CCT GGT 3'

JMB 4

3' CGG GGA GGA AAA CTC TCT TTG AGG GGA CCA 5'

Figure 6.2 Synthetic oligonucleotides constructed on the basis of known protein sequence. JMB 1 and 3 are oriented in the sense direction (5' - 3'), while JMB 2 and 4 are oriented in the antisense direction (3' - 5').

(section 6.2.6) while those in the antisense direction were constructed for use in northern blotting and library screening (section 6.2.5).

Codon choice was influenced by several important parameters. Since different mammalian tissues display different patterns of codon usage, codon utilisation in genes coding for other caprine milk proteins was taken into consideration (section 6.2.2). The sequence CpG is under-represented in mammalian DNA (Bird, 1980), therefore where a combination of codons generated this sequence, one codon was replaced with the next most commonly used. Additionally when there was no strong preference for any particular codon, those ending in T were picked.

6.2.4 Library construction

An oligo dT primed goat mammary library was constructed as described in section 2.8. A brief scheme is presented below. Total RNA was isolated from lactating goat mammary tissue as described in section 2.2.2. Messenger RNA (mRNA) was obtained from total RNA using a Poly Attract mRNA isolation system (section 2.8.1). Virtually all eukaryotic mRNAs have 3' poly (A) sequences allowing isolation of mRNA from total RNA by virtue of the mRNA's ability to bind oligo dT. First strand cDNA was synthesised following a modification of the method of Krug & Berger (1987) using MMLV reverse transcriptase and oligo dT (section 2.8.2). Double stranded cDNA was subsequently synthesised using RNAse H and DNA polymerase I, as described by Gubler (1987; see section 2.8.3). Following purification by phenol:chloroform extraction, the double stranded molecule was further purified, on the basis of size, by

centrifugation through a S300 spun column (section 2.8.5). Blunt ended cDNA was prepared for insertion into λgt11 by addition of ECoR I/Not I adaptors (section 2.8.6). Excess adaptors were removed by centrifugation through a spun column as before (section 2.8.5), and the cDNA was inserted into a λgt11 vector as described in section 2.8.7.

Several specific problems were encountered during library construction. First strand synthesis was generally successful. However second strand synthesis was inefficient, resulting in double stranded molecules that may have contained gaps or traces of mRNA. First and second strand synthesis were repeated many times to try to solve this problem. In addition the final library contained both shorter and fewer inserts than desired. Size separation using S300 spun columns was repeated to try to remove small fragments from the library, but this was only partially successful. The small average insert size was probably due to inefficiency of second strand synthesis. The constructed library, though not without its problems, was amplified ready for screening with either oligonucleotide probes or PCR products from 3' RACE.

6.2.5 Hybridisation with synthetic oligonucleotide probes

Oligonucleotides JMB 2 and JMB 4 were labelled at the 5' end with [γ-^{32}P]ATP using T4 polynucleotide kinase as described in section 2.8.2. Hybridisations were carried out as described in section 2.8.3, using northern blots of the RNA used for library construction. The choice of hybridisation temperature is crucial to the success of oligonucleotide probes - the temperature must be high enough to avoid non-specific

binding, but low enough to maximise the probability of specific binding. Hybridisation with synthetic oligonucleotides is generally carried out at temperatures 5 - 10 ° C below the calculated melting temperature (T_m) of a perfect hybrid. The T_m of each oligo was determined using a Genequant calculator (Pharmacia) according to manufacturer's instructions. The T_m of JMB 2 was calculated as 75.8 ° C and that of JMB 4 as 77.8 ° C. The maximum possible mismatch was also calculated, assuming that all choices of degenerate codons were incorrect. Both oligonucleotides had a percentage mismatch of 13 %. 1 ° C was subtracted from the calculated T_m for each 1% of mismatch; the resultant T_m should be that of a maximally mismatched hybrid formed between the guessmer and its target sequence. In practise the actual T_m should be higher than this worst case scenario, since even some randomly chosen codons could in fact be correct, however it is best to hybridise at a temperature lower than the estimated T_m to avoid missing the clone of interest.

Prior to library screening, a set of trial experiments were performed in which a series of northern hybridisations were carried out with varying T_m and washing conditions in order to optimise hybridisation conditions. Trial hybridisations were performed with two identical blots containing the same RNA as was used for library construction. Each blot was treated stepwise but asynchronously, such that loss of signal with any temperature increment could be corrected by a smaller increment with the second blot. Accordingly, hybridisation in aqueous buffer (section 2.9.3) was started at 50 ° C for each guessmer. After hybridisation, each blot was washed using 5 ° C increments in washing temperature, starting with the same conditions of temperature and ionic

strength as the hybridisation buffer. Hybridisation was repeated using the same hybridisation conditions but washing blots at conditions of higher stringency - *i.e.* at higher ionic strengths (section 2.9.3).

A similar procedure was carried out for hybridisation in formamide hybridisation buffer. This hybridisation buffer (section 2.2.4) contains 50% formamide. Each percent of formamide present in a hybridisation buffer is equivalent to a temperature increase of 0.5 ° C, therefore hybridisation in this buffer should be carried out 25 ° C lower than hybridisation in aqueous buffer. Each oligonucleotide was therefore hybridised in this buffer at 25 ° C. Washing conditions were as described previously.

Despite performing a number of northern hybridisations, no binding of either JMB 2 or 4 to RNA used for library construction was observed. Since no sequences complementary to either oligonucleotide probe were found it was decided not to screen the oligo dT primed library with either probe at this time.

6.2.6 3' RACE

3' RACE was performed as described in section 2.9.4. RACE generates cDNAs using the polymerase chain reaction to amplify copies of the region between a single point in the transcript and the 3' or 5' end. The sequences of the adapter primer and the oligo dT - adapter primer are shown in figure 6.3. No PCR products were obtained using either primer JMB 1 or 3.

dT_{17}-adapter primer

5' GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT 3'

Adapter primer

5' GAC TCG AGT CGA CAT CG 3'

Figure 6.3 Synthetic oligonucleotide primers for 3' RACE.

6.3 DISCUSSION

Attempts to clone the gene for caprine FIL were unsuccessful. This chapter highlights some of the problems experienced in the cloning of genes in general, and demonstrates that despite the use of a number of cloning strategies, success does not necessarily follow.

Guessmers were first used to identify bovine trypsin inhibitor (Anderson & Kingston, 1983), human insulin-like growth factor I (Ullrich *et al.*, 1984) and human factor IX (Jaye *et al.*, 1983). Many other genes have been identified by this method (reviewed by Maniatis, 1989) but there is as yet no report of a milk protein gene being isolated in this fashion.

Prior to library screening, a set of trial experiments were performed in which a series of northern hybridisations were carried out under different washing conditions and degrees of stringency. No sequences complementary to either oligonucleotide probe were observed in hybridisations with RNA used for library construction. It was therefore unlikely that any complementary sequences would be identified during library screening. This strategy was discounted at this stage.

In 3' RACE a short stretch of sequence from an exon must be known. From this region a gene specific primer oriented in the 3' direction is designed, providing specificity to the amplification step. RNA is reverse transcribed using a primer which consists of oligo dT (17 residues) attached to a unique 17 base "adapter" primer.

Amplification then takes place using the adapter primer, which binds to the cDNA at the 3' end, and the gene-specific primer (either JMB 1 or 3). RACE may have suffered from the same limitations experienced with oligonucleotide screening. The degeneracy of the primers designed for use in 3' RACE may also have been too high for any match to occur. The results obtained during trial northern hybridisations (section 6.2.5), where neither oligonucleotide bound to RNA, suggest that this was indeed the case. On the other hand, only one set of conditions was used for RACE, and while these conditions have been used successfully to clone other genes (Frohman *et al.*, 1988), they may not have been optimal for the current study.

These results suggest that the degeneracy of the oligonucleotides was too high for any match to occur. The codons chosen for each amino acid may not have been correct especially since there was a high proportion of amino acids in the partial protein sequence with four or six codons coding for the amino acid; for example alanine, glycine and proline with four codons, and serine and arginine with six possible codon choices.

Caprine FIL has proved hard to purify in a form suitable for protein sequencing, and the protein sequence published by Wilde *et al.* (1995) is a consensus sequence from several sequence attempts. An error in identifying even a single amino acid, combined with the high degree of codon degeneracy, could together have prevented identification of complementary sequences in PCR and library screening.

Structural analysis of FIL and cloning of the FIL gene is currently concentrating on the bovine form of the protein. The bovine protein has proved easier to purify and work is currently ongoing towards cloning the gene from this animal. If bovine FIL is identified a cDNA probe derived from this may facilitate identification of the gene for caprine FIL.

Chapter Seven

Summary and discussion

7.1 SUMMARY AND DISCUSSION

This study has increased knowledge of the role of FIL in the regulation of milk protein synthesis and secretion by showing first, that frequency or completeness of milk removal regulates the expression of milk protein genes and, secondly that this effect is mediated by FIL.

Previous evidence for regulation of mammary differentiation by milking frequency rested on measurements of lipogenic mRNA abundance and activity (Travers & Barber, 1993; Wilde *et al.*, 1987b). In extending these observations to two key milk protein genes, α-lactalbumin and α_{s1}-casein, we have confirmed that autocrine regulation is exerted on aqueous and non-aqueous milk constituents, *i.e.* that the developmental response is indeed one of cellular differentiation for secretory function. One of the milk constituents studied, alpha-lactalbumin, is an essential component of lactose synthase, the rate-limiting enzyme involved in the synthesis of lactose and therefore an important determinant of milk volume (Stacey *et al.*, 1995). Thus, as the rate of milk secretion increases in response to more frequent milk removal, milk composition remains unaltered. This suggests that individual milk constituents are indeed regulated coordinately.

A number of technical problems were experienced in the studies *in vivo*. For example, the response of individual animals to unilateral frequent milking, suckling or drainage was highly variable, making statistical analysis difficult. The problem could, of course, have been alleviated by use of a greater number of animals but this option was not available.

Analysis was also complicated by longitudinal changes in gene expression with stage of lactation. Consequently, a response to manipulation of milk removal was sometimes apparent as an accelerated or an impaired developmental response in the test gland, and not necessarily as a significant difference between glands after manipulation (Travers & Barber 1993; Wilde *et al.*, 1987b; Wilde *et al.*, 1990). In one case, the experiment was also confounded by practical problems. The response of the gland to continuous milk removal (drainage) was inconsistent. The catheters inserted in the gland frequently became dislodged or blocked, leading to discontinuities in the rate of milk removal. Additionally, catheter milking removes only cisternal milk and therefore fails to relieve autocrine inhibition (Henderson & Peaker, 1987). Alveolar milk was removed only once daily when the gland was milked out using oxytocin. This treatment may be considered a negative control - it confirms that effective manipulation of milk removal is absolutely essential for changes in both milk yield and milk protein gene expression.

Experiments in chapter four provided the first direct link between FIL and regulation of gene expression. The EHS culture system provides a convenient means of studying and manipulating milk protein gene expression. Murine mammary epithelial cells cultured on EHS matrix show a greater differentiated function than those cultured on either plastic or collagen. In EHS culture, murine mammary cells express high levels of several milk protein genes: β-casein (Li et al., 1987), whey acidic protein (Chen & Bissell, 1989) and transferrin (Chen & Bissell, 1987). The current study demonstrates that caprine mammary epithelial cells, like their murine counterparts, express high levels of milk protein genes when cultured on EHS matrix, making this system appear ideal for investigation of FIL's effects on gene

expression. However, recent evidence from primary culture of mammary epithelial cells indicates that the receptor for FIL is located on the apical surface of the secretory cell (Blatchford & Wilde, unpublished observations). Therefore if lumen formation is successful, FIL introduced in the culture media will be unable to reach its apically located receptor. That gene expression was not affected following short-term (4 hour) exposure to FIL may have been due to the protein's inability to reach this receptor. The reduction in milk protein gene mRNA abundance seen in cultures exposed to FIL for extended periods (72 hours) suggest that with time FIL did indeed gain access to its site of action. Alternatively, FIL may have gained access to the luminal space before the formation of tight intercellular junctions.

That FIL is competent in mammosphere cell culture to decrease milk protein gene expression suggests that FIL regulates mammary differentiation. More frequent removal of milk, and thus more frequent removal of FIL from the gland may therefore be expected to increase milk protein mRNA abundance. As was demonstrated in chapter 3, this is indeed the case.

A variety of standard methods were attempted in order to clone the gene for the inhibitory protein. That none was successful could stem from a fundamental technical problem - for example the purity of the FIL protein which was sequenced and used for antibody production. However, immunoblotting confirmed the specificity of the anti-FIL antibody prior to library screening, suggesting that cDNA for FIL, if present, should have been identified using this approach (Wilde *et al.*, 1995 & this study). Alternatively lack of

success in cloning may arise from a series of unrelated factors. It is possible, due to problems with the random primed library, that the antigenic portion of FIL was not represented. In addition the identification of a protein antigenically similar but unrelated in structure or function to the protein of interest has been experienced by other researchers (Boutin *et al.*, 1988; Cowell & Hurst 1992). Lack of success with 3' RACE was most likely due to the degeneracy of the oligonucleotides used. The goat displayed no strong codon bias, making construction of guessmers difficult (chapter six).

The bovine form of the protein has proved easier to purify and work is currently ongoing to clone the gene from this animal. If the gene for the bovine feedback inhibitor of lactation is identified, a cDNA probe derived from this may facilitate identification of the caprine form of the gene. Indeed, identification and characterisation of the bovine form will allow many of the questions asked in this thesis to be answered, in particular how FIL's milk concentration is controlled, and how FIL expression is regulated by FIL itself.

Access to a recombinant gene is also an essential step in the production of recombinant protein. It is possible that recombinant protein production may be achieved in either a prokaryotic or eukaryotic system; however this will depend on whether FIL is active in unglycosylated form. The availability of a bioactive recombinant protein would obviate many of the practical restraints which have thus far prevented elucidation of FIL's physiological significance and detailed examination of the molecular mechanism of autocrine control.

Autocrine control of milk production by FIL provides a plausible explanation for the matching of milk supply with demand. However, feedback control of milk secretion by FIL cannot act in isolation - it must be integrated with the endocrine control of lactation. Therefore, while focusing on the local autocrine regulation of milk secretion, it must be remembered that this mechanism is likely to be primarily a tactical device for matching supply of milk with demand, and that it operates within the strategic limits set by the endocrine system.

The dynamic nature of the mammary gland allows it to adapt to the demands placed on it by the suckled offspring or in dairy animals, by the milking regimen. This offers potential for controlling lactation in order to improve animal productivity and welfare. For example, identification of the gene for FIL, the feedback inhibitor of lactation, and elucidation of its role in milk secretion may allow milk secretion to be controlled by means other than those which led to the discovery of this protein, *i.e.* by frequency of milking. The ability to inhibit dramatically or switch off lactation by FIL treatment may in addition offer an adjunct to dry cow therapy - it may allow lactation to be switched off without mammary distension and the resultant discomfort this causes the animal. Accumulation of milk within the gland at drying off plays a major contribution to the incidence of mastitis in the dairy cow - a disease which costs the dairy industry millions of pounds annually.

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