Prolactin-induced proteins and dependence on protein synthesis for β-casein expression in mouse mammary cells

by

Angela Beaton

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Abstract

The regulation of milk protein gene expression in mammary epithelial cells occurs in response to multiple hormonal signals, plus cell-matrix and cell-cell interactions. A number of signalling molecules have been implicated in this process, but the exact molecular mechanisms regulating milk protein gene expression remain to be established. The project described in this thesis focuses on investigating the *de novo* synthesis of proteins in response to prolactin in mammary epithelial cells, to determine the role of *de novo* synthesis in lactogenic signalling, and to identify proteins which are induced by prolactin that could act as mediators of lactogenic signals. Two main approaches were taken:

(1) The results of proteome analyses using two-dimensional electrophoresis revealed a number of prolactin-responsive proteins in cells from the mouse mammary epithelial cell line, COMMA-D, and also lactation-associated proteins in freshly isolated mouse mammary epithelial cells. Two prolactin-induced and lactation-associated proteins, p77 and p63, were identified as glucose regulated protein 78 (grp78) and protein disulphide isomerase (PDI), and assessed as potential regulators of lactation. These proteins are reticuloplasmins and probably function in mammary epithelial cells in the processing of milk proteins for secretion. The results of this investigation have shown that grp78 and PDI were not rapidly induced in response to prolactin, thus it is unlikely that these proteins are involved in mediating lactogenic signals in response to prolactin.

(2) The requirement for *de novo* protein synthesis for maximal prolactin-stimulated β-casein gene expression was evaluated in COMMA-D cells using the protein synthesis inhibitors cycloheximide (CHX) and anisomycin. It appears that protein synthesis is required at the level of transcription for maximal induction and maintenance of β-casein gene expression (over the first 8 h of prolactin treatment). Furthermore, this effect was shown to be very rapid and independent of the lactogenic hormones, insulin and hydrocortisone, and the extracellular matrix. To determine the molecular basis for the effect of CHX on β-casein mRNA levels, STAT5 protein levels and DNA-binding activity were analysed in COMMA-D cells cultured in the presence or absence of prolactin and/or CHX. The results of Western analyses showed that STAT5 protein levels were not significantly altered in

the presence or absence of (prolactin and/or) CHX. A DNA probe, denoted STAT5(30), derived from the rat β-casein promoter sequence (-104 to -75), which contains a high affinity STAT5 consensus sequence was used for EMSA. CHX treatment stimulated the induction of a DNA-binding activity of distinct mobility to STAT5. This protein-DNA complex was shown to contain the p50 subunit of NF-κB. Detailed analysis revealed that CHX-stimulated NF-κB bound to a putative κB half-site on the STAT5(30) probe (GGAATT), which overlaps the high-affinity STAT5 DNA-binding site (TTCTTGGAA). Therefore, the effect of CHX appears to be mediated by inhibition of IκB synthesis, leading to the activation of NF-κB. Since the κB site overlaps the STAT5 binding site, it is possible that NF-κB competes for binding to the β-casein promoter and sterically hinders STAT5 binding and transactivation of β-casein transcription. It is conceivable that competition between NF-κB and STAT5 is functionally significant if NF-κB is activated in response to physiological signals in the mammary gland. This remains to be demonstrated.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

S.I. (Système International d'Unités) abbreviations for units, and standard notations for chemical elements, formulae and chemical abbreviations are used in the text. Other abbreviations are listed below.

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
APS	ammonium persulphate
ATP	adenosine triphosphate
BCE-1	bovine β-casein enhancer-1
BiP	immunoglobulin heavy chain binding protein
°C	degrees Celcius
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer-binding protein
CHX	cycloheximide
CIDR	controlled intravaginal drug release device
CIS	cytokine-inducible SH2-containing
cpm	counts per minute
d	day
DAB	3,3'-diaminobenzidine tetrahydrochloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMEM:F12	Dulbecco's modified Eagle's medium F12 nutrient mix
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRB	5,6-dichlorobenzimidazole
DTT	1,4-dithiothreitol
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
GAS	γ-interferon-activated sequence
GR	glucocorticoid receptor
Grb2	growth factor receptor bound 2
grp78	glucose regulated protein 78
h	hour
HBP	helix bundle peptide
HPLC	high performance liquid chromatography

hsp90	heat shock protein 90
i.d.	internal diameter
IL	interleukin
IRS	insulin receptor substrate
JAB	JAK binding protein
JAK	Janus kinase
kb	kilobases
kDa	kilodaltons
LAP	liver-enriched activated protein
LIP	liver-enriched inhibitory protein
mA	milliamperes
MAPK	mitogen-activated protein kinase
min	minutes
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MW	molecular weight
NF-1	nuclear factor 1
NP-40	Nonidet P-40
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDI	protein disulphide isomerase
pI	isoelectric point
PI 3-kinase	phosphatidyl inositol 3-kinase
PRL	prolactin
PRL-R	prolactin receptor
RNA	ribonucleic acid
RT	room temperature
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SH2	*src* homology 2
SHC	*src* homology collagen
SOCS	suppressors of cytokine signalling
Sos	Son-of-sevenless
SSC	salt/sodium citrate
SSI	STAT-inducible STAT inhibitor
STAT	signal transducer and activator of transcription
T_3	triiodothyronine
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetra-methylenediamine
Tris	tris(hydroxymethyl)aminomethane
V	volts
v/v	volume per volume
w/v	weight per volume
WAP	whey acidic protein
YY1	Yin-Yang 1

Chapter One - Literature Review

THE MAMMARY GLAND

(i) Milk composition -

A distinctive feature of milk lies in its ability to satisfy two important biological functions in mammals. First, it provides the sole form of nutrition to the neonate and, second, it confers immunological protection via the transfer of immunoglobulins in colostrum (Jenness, 1974). Consequently, milk contains a wide array of molecules, including water, fats, proteins, carbohydrates and minerals. The exact levels of these components vary depending upon the species, the stage of maturity of the newborn, the stage of lactation, environmental conditions and the diet of the lactating animal (Collier, 1985). Milk fats are the most variable component of milk, in terms of both their chemical composition and concentration. Dietary lipids act in structural, metabolic and storage roles (Mepham, 1987), and milk fat is also an important source of fat-soluble vitamins such as A, D, E and K. However, despite the nutritional properties of milk, there is some concern about the possible health risks associated with the consumption of animal-derived fats. Thus, the fat composition and protein/fat ratio in milk are potential targets for manipulation.

There are two main classes of milk proteins, the caseins and the whey proteins. The caseins are precipitated at acid pH (4.6 for bovine casein, the isoelectric point), or by the action of the gastric enzyme chymosin/rennin. The caseins are phosphoproteins that form calcium-dependent micelles via their interaction with the phosphoglycoprotein κ-casein (Rollema, 1992; Duncan & Burgoyne, 1996). The presence of κ-casein is significant since it is

hydrolysed by chymosin to form milk clots, aiding in digestion in the neonate and in the development of the coagulum in cheese making. The caseins are the most abundant milk proteins and are classified into several types, αS-, β-, δ- and κ- (in cows milk). However, molecules within a particular type or class are also subject to genetic variation and different degrees of post-translational modification.

Milk whey proteins remain in solution at a pH which precipitates the caseins. The two main whey proteins present in milk are α-lactalbumin and β-lactoglobulin. α-lactalbumin is present in milk from most species, and has an important role in lactose biosynthesis (Brew & Grobler, 1992). In mammary cells, galactosyltransferase is bound to the inner surface of the Golgi, where it temporarily interacts with α-lactalbumin, as it is transported with other secretory proteins to the apical membrane for secretion. α-lactalbumin interacts with galactosyltransferase to greatly enhance its affinity for glucose, and form the functional lactose synthetase complex. Lactose is synthesised in the lumen of the Golgi, and is confined to either the Golgi or secretory vesicles, where it accumulates and acts as an osmole to help generate the aqueous phase of milk. Since milk has a high water content, lactose production is crucial for the secretion of milk.

β-lactoglobulin is the major whey protein in the milk of ruminants, but it is absent in the milk of humans and rodents. The biological function of β-lactoglobulin remains uncertain (Hambling *et al.*, 1992). β-lactoglobulin has a high nutritive value; however, it may also play another role through its acid stability and ability to bind ligands such as fatty acids and vitamin A.

(ii) Mammary gland anatomy and physiology -

There are marked differences between species in the number and anatomical position of mammary glands, however their histological structure is remarkably uniform. In common with all exocrine glands, the parenchyma

of the mammary gland consists of two types of tissue, secretory and ductal, arranged in structures which undergo cyclical changes depending on the stage of oestrous or lactation. Mammary glands undergo repeated cycles of growth, differentiation and regression, ultimately passing through a number of developmental and functional stages. Each stage is regulated by complex interactions between a number of hormones. Some of the hormones known to influence mammary function are listed in **Table 1**.

From birth to puberty, in most mammalian species the mammary gland basically consists of a nipple and a rudimentary duct system extending throughout an adipose stroma (or fat pad). The adipose stroma of the mammary gland is composed of mesenchymal precursors, adipocytes, fibroblasts, interstitial collagen, blood and lymph vessels and nervous tissue, and supports the parenchyma in both structure and function (for a review see Neville *et al.*, 1998). At this stage, the ducts terminate in endbuds, specialised groups of cells which are sites for proliferation in the growing and developing gland.

Mammary growth is isometric until puberty, at which time, there is considerable ductal growth throughout the mammary fat pad in response to enhanced secretion of oestrogen. The critical role of oestrogen in regulating mammary development was confirmed by showing that in rodents ovariectomy before puberty suppresses mammary development, and that this is reversible by the administration of exogenous oestrogen (Flux, 1954). It is thought that oestrogen synergises with thyroid hormones (Lyons, 1958), and that insulin and cortisol may also play a role. In addition, a range of growth factors including insulin-like growth factor-1, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor, pituitary-derived mammary growth factor and the transforming growth factors α and β are also involved in the hormonal regulation of mammary gland development (Oka *et al.*, 1991).

Table 1 *Hormones involved in regulating mammary development and lactation*

a) Development		
Foetal	*Puberty*	*Pregnancy*
Androgens inhibit	Oestrogen	Oestrogen
	Growth hormone	Progesterone
	Triiodothyronine (T_3)	Growth hormone
	Insulin	Prolactin
	Growth factors	Cortisol
		T_3
		Insulin
		Growth factors
b) Lactation		
Initiation	*Maintenance*	*Milk Letdown*
Oestrogen withdrawl	Prolactin	Oxytocin
Progesterone withdrawl	Insulin	
Prolactin	Cortisol	
Cortisol	T_3	

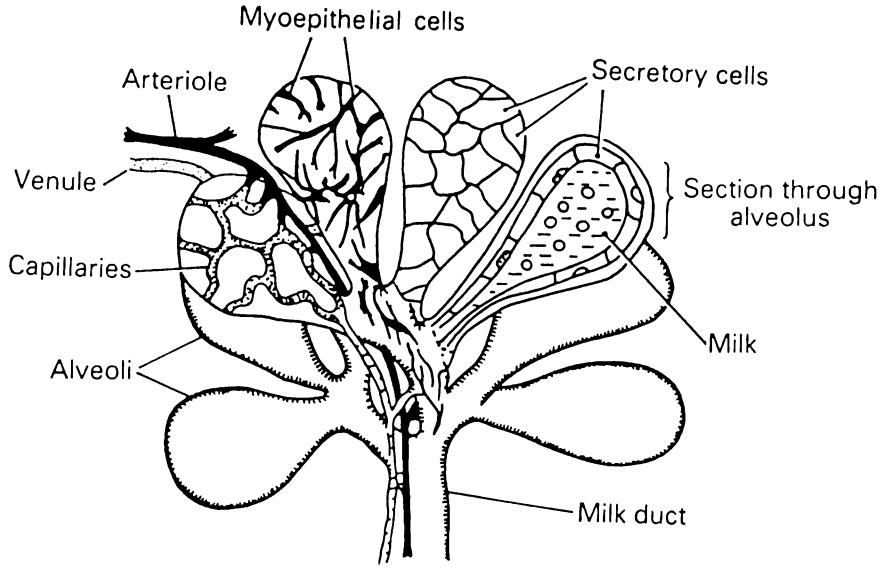
adapted from Rillema (1994).

During pregnancy, mammary cell structural differentiation occurs as a defined sequence of events. During the early stages of pregnancy, ductal and alveolar mammary epithelial cells proliferate in response to elevated levels of oestrogen and progesterone derived from the ovary and/or the placenta (depending on the species). Epithelial-stromal interactions are critical for the initiation and maintenance of oestrogen and/or progesterone responsiveness in mammary epithelial cells both *in vitro* and *in vivo* (Haslam, 1986; Haslam & Counterman, 1991). The final stage of mammary development is termed terminal differentiation, and results in the emergence of secretory epithelial cells (Topper & Freeman, 1980). In conjunction with this, mammary fat pads regress and vascularisation of the gland increases, to form a highly developed structure distinct from that seen at any other developmental stage. Lactogenesis is a precipitous event, and although the exact

mechanisms controlling the initiation of lactation are not known, many endocrine changes are known to occur. Progesterone withdrawl, increased concentrations of prolactin, cortisol and oestrogen in blood, parturition and suckling, are all considered to be important influences during lactogenesis (Kuhn, 1977; Mepham, 1987). Species differences also add to the complexity of this process. For example, growth hormone is a potent lactogenic hormone in ruminants, but has little effect in rodents (for a review see Burton, 1994).

In the lactating mammary gland, secretory tissue is composed of specialised secretory epithelial cells grouped in structures called alveoli. An individual alveolus consists of a single layer of epithelial cells which secrete their products into a central lumen (see **Fig. 1**). Alveoli are surrounded by myoepithelial cells which are involved in milk ejection and blood vessels that supply nutrients, oxygen and hormonal signals to the cells. During lactation, myoepithelial cells contract in response to oxytocin, forcing the alveoli to release milk from the lumen and into the ductules. Milk products collect through ductules into the duct system, and groups of alveoli drained via a common duct constitute a lobule.

Secretory cells are polarised with a nucleus situated toward the basal side of the cell (Larson, 1985). Milk precursors are taken into the cells from the blood through the basal and lateral membranes and milk is secreted into the lumen through the apical membrane. Tight junctional complexes located along the apical portions of the cells form the barrier between blood and milk. Mammary epithelial cells contain a well developed rough endoplasmic reticulum and Golgi apparatus, a feature common to secretory cells, as well as a large number of mitochondria and lysosomes. Lactation is characterised by copious milk synthesis and secretion and continues for an extended period of time depending on the species. A number of hormones, including insulin, glucocorticoids, thyroid hormones and prolactin, are thought to be important for the maintenance of lactation (for reviews see Delouis, 1980; Rillema, 1994).



adapted from Mepham, (1987).

Figure 1 *Diagrammatic representation of a cluster of mammary alveoli.*

On weaning or the cessation of milk removal, the mammary gland becomes swollen with milk, milk components are no longer secreted, milk stasis occurs, and levels of systemic lactogenic hormones fall (Hollmann, 1974; Feng *et al.*, 1995). As intramammary pressure increases in the gland, milk protein synthesis decreases with no initial change in cell morphology. However, if stasis persists, the secretory cells regress into a less differentiated state. This process is termed involution and involves both alveolar programmed cell death and lobulo-alveolar remodelling (for a review see Jaggi *et al.*, 1996). Mammary gland involution occurs in two distinct stages (Li *et al.*, 1997). The first stage is characterised by programmed cell death of alveolar cells and certain changes in gene expression, but no remodelling of the lobulo-alveolar structure (Marti *et al.*, 1994; Boudreau *et al.*, 1995). The second stage of involution, however, involves extensive tissue remodelling by proteinases which degrade the basement membrane and extracellular

matrix (Ossowski *et al.*, 1979; Strange *et al.*, 1992; Feng *et al.*, 1995; Lund *et al.*, 1996). This results in a gland with very small alveoli containing only a few cells and increased numbers of stromal cells (Wooding, 1977).

THE MAMMARY EPITHELIAL CELL

(i) In vitro models of mammary function -

In vitro cell culture systems have provided us with the means of studying the cellular events which trigger the induction of milk protein gene expression, while avoiding the substantial problems inherent with *in vivo* experiments with the mammary gland (for a review see Ip & Darcy, 1996). Whole gland culture, primary explants, primary cell culture and cell lines are commonly used as *in vitro* models of mammary function. Explant culture uses small pieces of isolated mammary tissue. As is the case with whole organ culture, this favours the retention of some of the epithelial-stromal interactions characteristic of the *in vivo* tissue, however maintaining viability of the cells without a blood supply to transport oxygen, nutrients and hormonal signals to every cell can be difficult. In addition, it is not always clear whether a modulator of mammary function exerts its effect directly on the mammary cell, or acts indirectly through the stroma.

Primary mammary epithelial cell culture involves culturing small clumps of cells which have been enzymatically digested from mammary tissue. When cultured on an extracellular matrix under the appropriate hormonal conditions, cells will maintain their differentiated phenotype. However, overcoming certain problems such as contamination from the growth of fibroblastic cells can be difficult. Finally, the establishment of mammary epithelial cell lines such as COMMA-D (Danielson *et al.*, 1984), derived from the mammary glands of mid-pregnant mice, and its clonal derivative HC-11 (Ball *et al.*, 1988), have provided valuable model systems. COMMA-D cells are a mixed population of cells within which only 10-20%

are capable of casein synthesis in response to hormonal stimuli. As with primary cell culture, these cells do not synthesise all of the milk proteins in response to hormonal stimulation (Medina, 1987).

Clearly, no single *in vitro* system is ideal as a model of mammary function, so the choice of a system must depend on the question being asked. Nevertheless, from the results of *in vitro* studies, it has been established that hormonal signals, cell-cell and cell-matrix interactions are critical if mammary epithelial cells are to differentiate and synthesise the major components of milk (Emerman & Pitelka, 1977; Emerman *et al.*, 1977).

(ii) Hormonal regulation of lactation -

Prolactin is known to be involved in regulating a large number of diverse physiological processes including water and electrolyte balance, growth and development, metabolism and behaviour, immune responses and reproduction. However, prolactin is probably best known for its role in the mammary gland. Although a milieu of hormones are thought to regulate each developmental state, prolactin is the defining hormone in most species, stimulating lobulo-alveolar growth during pregnancy (Lyons, 1958), and the synthesis of the major components of milk, milk proteins (Lee *et al.*, 1984; 1985), lactose (Oppat & Rillema, 1988) and lipids (Waters & Rillema, 1988).

The anterior pituitary hormone prolactin belongs to the helix bundle peptide (HBP) hormone family. This family also includes growth hormone and placental lactogen, the interleukins, granulocyte-macrophage colony stimulating factor and erythropoietin. While various functions have been ascribed to individual hormones in this family, the HBP hormones have broad overlapping physiological and molecular actions in many tissues (for a review see Horseman & Yu-Lee, 1994). Prolactin is a 23 kDa peptide hormone and is present in all vertebrates, and cDNAs encoding prolactin from several species have been cloned and sequenced (Cooke et al., 1980;

1981). Prolactin varies in size from 197-199 amino acids between species and contains six cysteines which form intramolecular disulfide bonds (cys 4-11, 58-174 and 191-199 in human PRL). Based on the significant sequence similarity that prolactin shares with growth hormone and placental lactogen, it is thought that these genes probably evolved from a common ancestral gene (Cooke *et al.*, 1981).

Prolactin is predominantly synthesised in the lactotrophic cells of the anterior pituitary, but is now known to be synthesised in cells and tissues outside the pituitary, including the mammary gland (reviewed by Ben-Johnathan *et al.*, 1996). This is important because extrapituitary prolactin may exert autocrine and/or paracrine effects on a tissue (Clevenger & Plank, 1997), without affecting the circulating levels of prolactin. Interestingly, a case of a woman with idiopathic prolactin deficiency and otherwise normal pituitary function has been reported (Kauppila *et al.*, 1987). Despite the multiple actions associated with prolactin, this disorder was first manifested with postpartum alactogenesis, confirming that prolactin is necessary for puerperal lactation. Thus, it is possible that in this case, extrapituitary prolactin, and/or the overlapping functions of other HBP hormones may be sufficient to regulate other prolactin-dependent physiological processes in other cells and tissues.

In addition to prolactin, insulin and glucocorticoids can also function as lactogenic hormones, and have been implicated in the activation of milk protein genes, such as β-casein (Guyette *et al.*, 1979; Houdebine *et al.*, 1985; Wartmann *et al.*, 1996). The exact role of insulin in mammary epithelial cells has not been established. Insulin is known to elicit a wide variety of biological responses in many tissues and cell types, influencing several metabolic processes affecting glucose, lipid and protein metabolism, to stimulate cell growth and differentiation (Saltiel *et al.*, 1996). Insulin may play a similar role in mammary epithelial cells. In contrast, glucocorticoids are known to act synergistically with prolactin to enhance β-casein gene

expression and stabilise β-casein transcripts (Houdebine *et al.*, 1978; Doppler *et al.*, 1990). Glucocorticoids have been shown to stimulate development of the Golgi and rough endoplasmic reticulum in mammary epithelial cells derived from mid-pregnant mice (Topper & Oka, 1974; Tucker, 1985).

(iii) The influence of the extracellular matrix -

Clearly, hormonal signals have a major role in the regulation of milk protein gene expression, but it has also been established that cell-cell and cell-matrix interactions are critical aspects of mammary differentiation. The extracellular matrix (ECM) is comprised of the basal and reticular laminae (which act as a functional unit), and forms the acellular material connecting cells. The basal lamina contains a number of proteins, including laminin, type IV collagen, proteoglycans and glycoproteins, and separates the epithelial and ductal cells from the underlying stroma (Bissell & Hall, 1987).

The ECM is very important for the provision of a structural framework that enables cells to maintain the appropriate functional activity both *in vivo* (Bissell & Hall, 1987) and *in vitro* (Aggeler *et al.*, 1988). For example, mammary epithelial cells in the mid-late pregnant gland transcribe milk protein genes. However, if these cells are cultured, they will lose their differentiated phenotype and ability to transcribe milk protein genes, even in the presence of the three essential lactogenic hormones, insulin, hydrocortisone and prolactin. In contrast, if mammary epithelial cells derived from mid-pregnant mice are cultured on an appropriate ECM, they do not lose their differentiated characteristics as they do when cultured on plastic (Emerman & Pitelka, 1977). Instead, when cultured under appropriate hormonal conditions, the cells will re-arrange themselves *in vitro* to form secretory alveolar-like structures (Wicha *et al.*, 1982; Li *et al.*, 1987). This occurs by a process very similar to differentiation *in vivo*. Immediately after plating, cells are largely disorganised, but by the second or third day in culture cytoplasmic polarisation is observed, apical junctional complexes are

formed, and the synthesis of milk protein mRNA and protein is initiated (Aggeler *et al.*, 1991).

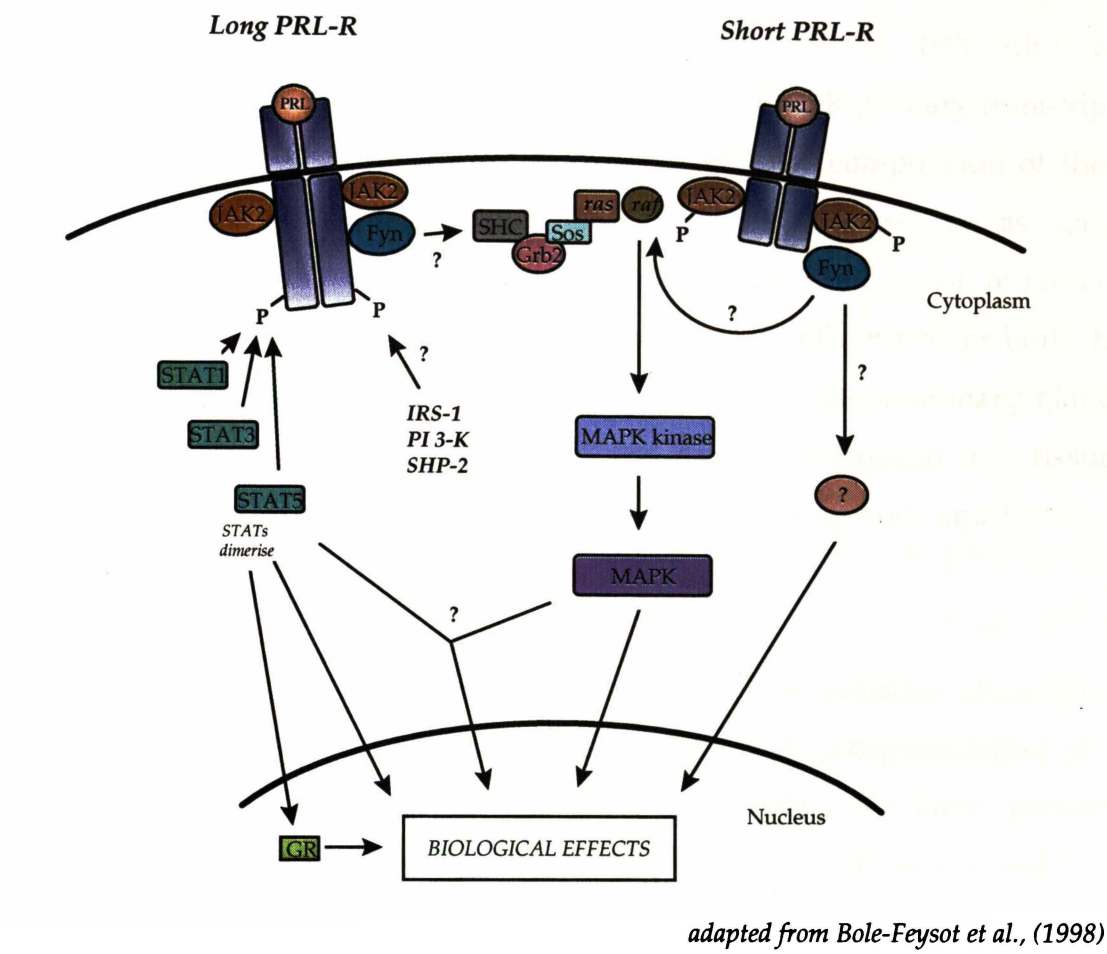
For cell culture, the reconstituted basement membrane derived from the Engelbreth-Holm-Swarm tumour, Matrigel®, is commonly used. This material contains laminin, type IV collagen, heparin sulphate proteoglycans and fibronectin, and closely resembles the basal lamina in contact with epithelial cells *in vivo*. From the results of cell culture experiments using Matrigel®, it has been established that one specific component of the extracellular matrix, laminin, is required for differentiation and is involved in mediating tissue-specific gene expression (Streuli *et al.*, 1995b; Streuli & Edwards, 1998).

(iv) Intracellular signalling pathways -

Prolactin signalling pathways

The responses to hormonal stimuli are mediated through multiple intracellular signalling pathways, which regulate the activity of genes controlling growth and differentiation. For prolactin, the cascades of intracellular signalling events activated by hormonal stimulation have been studied mainly in mammary epithelial cells, and the rat T lymphoma Nb2 cell line. A schematic representation summarising the intracellular signalling pathways stimulated by prolactin is shown in **Fig. 2**.

The prolactin receptor (PRL-R) represents the first step in the transduction of prolactin's signals from the cell membrane to the nucleus. In addition to the mammary gland, the PRL-R has been identified in a wide variety of tissues and cells, which reflects the wide range of effects mediated by this hormone.



adapted from Bole-Feysot et al., (1998)

Figure 2 *Schematic representation of the prolactin signalling pathways*

Long and short forms of the PRL-R are represented. The MAP kinase pathway is thought to be activated by both PRL-R isoforms and involves the SHC/Grb2/Sos/*ras*/*raf* cascade. The long form of the PRL-R also activates the JAK-STAT pathway, which results in the activation of STAT1, STAT3, and mainly STAT5. Whether the short PRL-R isoform activates the STAT pathway is currently unknown. Connections between the JAK-STAT and MAPK pathways have been suggested, but remain to be established. Interactions between the PRL receptors and *Src* kinases (eg. Fyn), SHP-2, IRS-1, PI 3-kinase, and other transducing molecules remain unclear. However, interaction between STAT5 and the glucocorticoid receptor (GR) has been reported (see main text). Grb2, growth factor receptor bound 2; IRS, insulin receptor substrate; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidyl inositol 3-kinase; PRL, prolactin; PRL-R, PRL receptor; SHC, *src* homology collagen; SHP-2, protein tyrosine phosphatase; Sos, Son-of-Sevenless; STAT, signal transducer and activator of transcription.

Multiple forms of the PRL-R have been identified in several species (Boutin *et al.*, 1988; 1989; Davis & Linzer, 1989; Shirota *et al.*, 1990; Ali *et al.*, 1991), which result from alternative splicing of the PRL-R primary transcript. The different PRL-R isoforms differ in the length and composition of their intracellular domains/cytoplasmic tails, and are referred to as long, intermediate and short forms. The intermediate form is a deletion mutant of the long form and is the predominant form in Nb2 cells, however both the long and the short forms of the PRL-R are present in the mammary gland. The different transcripts for the PRL-R are variably expressed in a tissue-specific manner, depending on the stage of oestrus, pregnancy and lactation (Nagano & Kelly, 1994; Clarke & Linzer, 1993).

The PRL-R has no intrinsic kinase activity, but prolactin stimulation leads to dimerisation of the receptor and the rapid phosphorylation of a number of cellular proteins (within 1 min), including the Janus protein-tyrosine kinase, JAK2, the mitogen-activated protein (MAP) kinase and Fyn, a member of the *Src* kinase family (Dusanter-Fourt *et al.*, 1994; Clevenger & Medaglia, 1994; Taniguchi, 1995; Das & Vonderhaar, 1996a; 1996b). Other cytokine receptors are also known to work in combination with the Janus protein-tyrosine kinase (JAK) family of proteins, which includes JAK1, JAK2, JAK3 and TYK2 (reviewed by Wilks & Harpur, 1994).

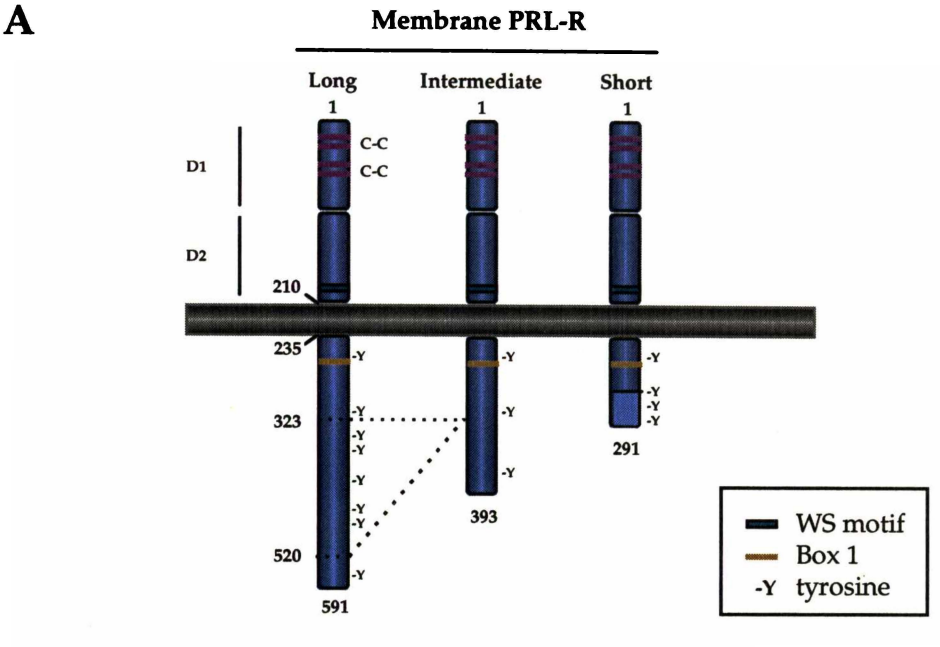
The PRL-R has been well characterised (reviewed by Goffin & Kelly, 1996; 1997; Bole-Feysot *et al.*, 1998), and putative functional domains for both the long and short forms of the PRL-R have been mapped (**Fig. 3**). The most membrane-proximal part of the intracellular domain is common to all of the receptors, and is required for interactions with the transducing molecules, JAK2, MAP kinase and Fyn, and the subsequent effects of prolactin on cell growth and milk protein gene transcription. JAK2 is constitutively associated with the PRL-R, and interactions between the receptor and JAK2 are facilitated by a proline-rich region (Box 1) found in the first 27 amino acids (Lebrun *et al.*, 1995). It is not known whether this interaction is mediated by an adaptor protein.

Figure 3 *The prolactin receptor (PRL-R)*

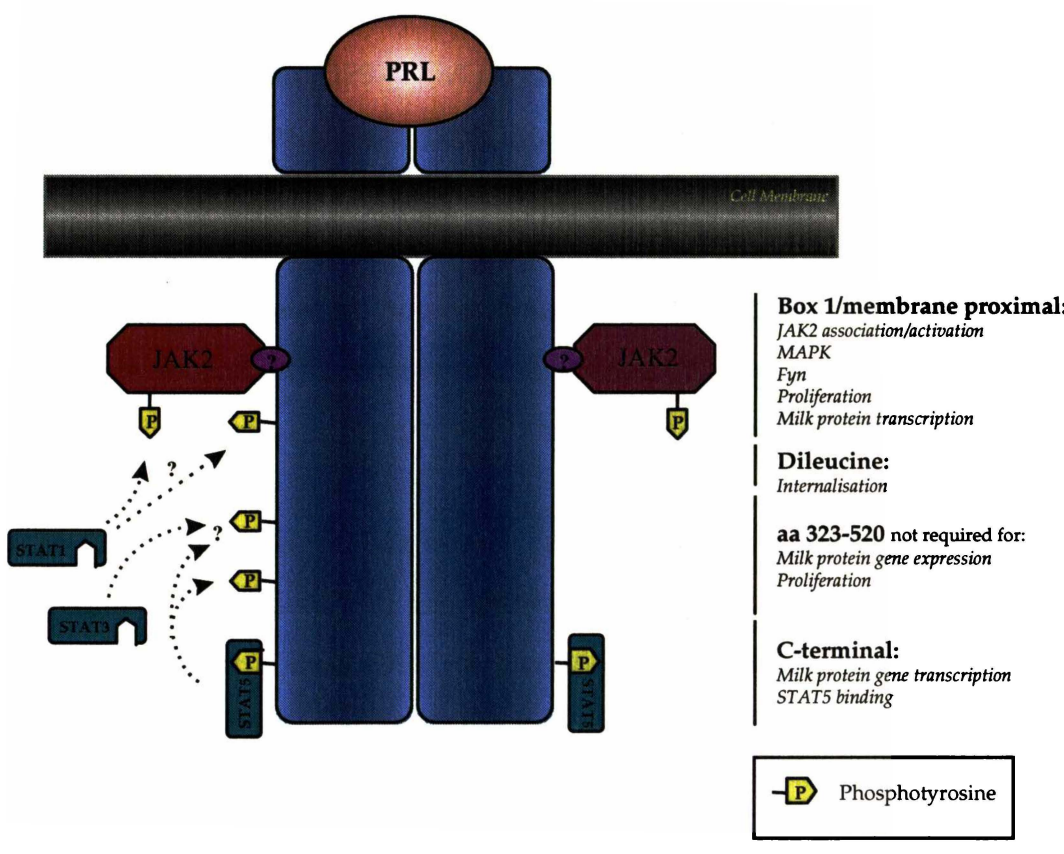
A) *Schematic representation of the long and short (rat) isoforms of the prolactin receptor (PRL-R).* All forms have identical extracellular, ligand-binding domains. Subdomain D1 contains pairs of disulphide bonded cysteines (C-C) and subdomain D2 contains the WS motif, two characteristic features of the cytokine receptor superfamily. Box 1 is found in the cytoplasmic domain of all membrane forms. In the rat, the intermediate form (only found in Nb2 cells), differs from the long form by a 198-aa deletion in the cytoplasmic domain (aa323-520). Otherwise, the short form is identical to both other forms up to residue 261, after which its sequence differs (*light blue box*). Cytoplasmic tyrosine residues are indicated.

B) *Structure-function relationships of the long PRL-R cytoplasmic domain.* The membrane-proximal region that is common to all PRL isoforms is required for interaction with and/or activation of JAK2, Fyn, and MAP kinases as well as for activation of cell proliferation and transcription of milk protein genes. Box 1 is required for JAK2 binding. The di-leucine motif (aa 259-260), identified in the short PRL-R is thought to be involved in internalisation of all PRL-R isoforms. Six tyrosines are potentially phosphorylated, but the most C-terminal (Y580) is required for STAT5 activation, and is proposed to be the major binding site of STAT5. STATs 1 and 3 are likely to interact with membrane-proximal regions of the receptor complex.

A



B



adapted from Bole-Feysot et al., (1998)

In addition, two motifs at positions 253-261 and 273-281 have been identified in the short form of the receptor, and are thought to be important for internalisation of all PRL receptors (Vincent *et al.*, 1997). Finally, a tyrosine residue present at position 580 is required for activation of the signal transducer and activator of transcription factor, STAT5, and is the major site of interaction between STAT5 and the PRL-R.

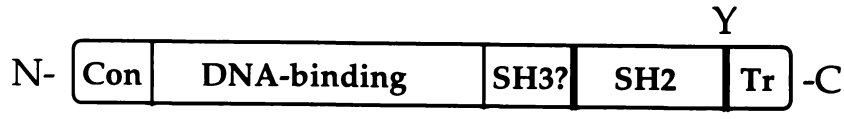
The precise roles for each form of the PRL-R remain unclear. Both forms are able to dimerise upon ligand binding and stimulate the activation of JAK2, Fyn, and MAP kinase to promote cell growth. While the long form of the receptor is also capable of transducing lactogenic signals to activate the β-casein promoter, the short form of the PRL-R is unable to do so, and has been shown to silence prolactin induction of the β-casein gene promoter (Berlanga *et al.*, 1997a). Thus, it is thought that the short form of the PRL-R is involved in mediating the mitogenic effects of prolactin to stimulate cell proliferation (Das & Vonderhaar, 1997). More recently, the role of the PRL-R has been investigated *in vivo* in mice carrying a germ line null mutation of the PRL-R gene (Ormandy *et al.*, 1997). Heterozygous females (PRL-$R^{+/-}$) showed an almost complete failure to lactate after the first, but not subsequent, pregnancies. However, homozygous females (PRL-$R^{-/-}$) were sterile due to multiple reproductive abnormalities. These results confirm the importance of the PRL-R for normal reproductive and mammary function.

Two major signalling routes from the cell membrane to the nucleus have been identified, and are known as the JAK-STAT and *ras/raf-1*/MAP kinase pathways (Schindler & Darnell, 1995; Das & Vonderhaar, 1996a; 1996b). The proposed model for prolactin signalling via the JAK-STAT pathway is initiated by the binding of prolactin to cell surface receptors. The activated receptors are phosphorylated on tyrosine by the associated JAK2, and the phosphorylated tyrosine then interacts with the STAT5 *src* homology (SH2) domain to form a PRL-R-JAK-STAT complex. STAT5 is phosphorylated by JAK2 and the phosphorylated STAT5 molecule

dissociates and forms homo- and heterodimers. Dimerised STAT5 then translocates to the nucleus to associate with specific DNA elements in the promoters of the milk protein genes. Thus, the STAT proteins perform a dual role in the JAK-STAT pathway (Ihle & Kerr, 1995; Horvath & Darnell, 1997). Firstly, as signal transducers by acting as substrates of the JAKs, and then as positively- or negatively-acting transcription factors (Bennett *et al.*, 1997; Luo & Yu-Lee, 1997).

To date, the STAT family includes STAT1 (α and β), STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6 and dSTAT, a STAT5 homologue found in *Drosophila*. The STAT proteins share several conserved structural and functional domains (summarised in **Fig. 4**), and are characterised by a DNA-binding domain, an SH3-like domain, an SH2 domain, a conserved tyrosine and a C-terminal *trans*-activating domain. The SH2 domain is involved in three key processes, the recruitment of STATs to activated receptor complexes, interaction with the JAKs, and STAT dimerisation and the associated ability to bind DNA (Heim *et al.*, 1995). STAT dimerisation is thought to be mediated by interaction between the phosphotyrosine on one molecule and the SH2 domain on the other. The SH3-like domains are less highly conserved and the exact function of these domains is currently unknown. STAT structure and function has been reviewed in more detail by Darnell, (1997).

The MAP kinase pathway is also stimulated by prolactin binding to cell surface receptors, and is thought to mediate the proliferative effects of the hormone (Das & Vonderhaar, 1996b; 1997). Signalling through the MAP kinase pathway involves the SHC (*Src* homology collagen)/ Sos (son-of-sevenless) /Grb2 (growth factor receptor bound 2)/p21 *ras*/*raf-1*/MAP kinase cascade. SHC proteins contain an SH2 domain. Upon tyrosine phosphorylation (by JAK2 in this case), SHC proteins act as adaptor molecules for other SH2-containing proteins in signal transduction pathways.



adapted from Ihle, (1996a).

Figure 4 *Schematic diagram illustrating the conserved functional domains of the STAT proteins.* The functional domains of the STATs are indicated, including a conserved region in the amino terminus (Con), the DNA-binding domain, a *src* homology domain 3-like region (SH3?), the highly conserved *src* homology domain 2 region (SH2), the critical site of tyrosine phosphorylation (Y), and the carboxy-terminal transcriptional activation domain (Tr).

For instance, Grb2 is a cellular substrate for SHC, and facilitates the formation of a SHC-Grb2-Sos complex. Sos is thought to be a guanine nucleotide exchange factor. Upon activation, Sos activates p21 *ras* which then, in turn, activates the serine/threonine kinase *raf-1* and the MAP kinase pathway. Prolactin has been shown to rapidly activate SHC proteins, *ras* and *raf-1*, and stimulate the association of the Grb2-Sos complex (Erwin *et al.*, 1995; Das & Vonderhaar, 1996a; 1996b). It has been suggested that connections may exist between the JAK-STAT and MAP kinase pathways, but this remains to be established (Ihle, 1996b).

A number of other potential signalling molecules have been identified. These include the insulin receptor substrate-1 (IRS-1), phosphatidyl inositol

3-kinase (PI 3-kinase), phospholipase C-gamma, protein kinase C, intracellular Ca^{2+} and members of the *Src* kinase family such as Fyn (Berlanga *et al.*, 1997b; Al-Sakkaf *et al.*, 1996; Buckley *et al.*, 1988; Berlanga *et al.*, 1995).

Protein-tyrosine phosphatases are known to modulate phosphorylation events during cytokine signalling. For instance, the protein-tyrosine phosphatase, SHP-2, positively regulates the activation of β-casein gene transcription in response to prolactin treatment (Ali *et al.*, 1996). It has recently been proposed that SHP-2 relieves an inhibitory tyrosine phosphorylation event in JAK2 required for its activity, STAT5 phosphorylation and transcriptional induction (Berchtold *et al.*, 1998). This contrasts with the role of other protein-tyrosine phosphatases in cytokine signalling, such as SHP-1, which negatively regulates signalling through the erythropoietin receptor (Klingmüller *et al.*, 1995).

Finally, a number of inhibitors of JAK/STAT signalling have recently been identified, which may regulate cytokine signalling in the mammary gland. These include JAB (a JAK binding protein), SSI (a STAT-inducible STAT inhibitor), and a family of cytokine-inducible, negative regulators of cytokine receptor signalling, which contains the CIS (cytokine-inducible SH2-containing) proteins and the SOCS (suppressors of cytokine signalling) proteins (Yoshimura *et al.*, 1995; Matsumoto *et al.*, 1997; Endo *et al.*, 1997; Naka *et al.*, 1997; Adams *et al.*, 1998).

Insulin and Glucocorticoid signalling pathways

Insulin exerts a wide range of effects in the cell through poorly defined signalling pathways. Although recently, some progress has been made with the identification of a number of cellular substrates of the insulin receptor. Upon activation the insulin receptor undergoes autophosphorylation on several tyrosine residues (White *et al.*, 1988), and transmits downstream signals via the phosphorylation of various cellular substrates. These include

IRS-1, IRS-2 and SHC (Sun *et al.*, 1991,1995; White, 1994; Pronk *et al.*, 1993). IRS protein-mediated signalling involves interactions between the IRS proteins and other SH2 domain-containing molecules (Myers & White, 1996). Among the proteins activated by IRS-1 are the Grb2-SOS complex and the protein-tyrosine phosphatase, SHP-2, which transmit mitogenic signals through the activation of *ras* (Ogawa *et al.*, 1998), and the PI 3-kinase, which elicits many of insulin's effects on glucose and lipid metabolism (Sheperd *et al.*, 1998). The lipid products of PI 3-kinase are thought to mediate insulin's effects by controlling the phosphorylation state of key regulatory metabolic enzymes (for a review see Stralfors, 1997). Finally, the recent identification of STAT5b as a substrate for the insulin receptor broadens the number of signalling pathways linking the activated insulin receptor to numerous downstream responses (Chen *et al.*, 1997; Sawka-Verhelle *et al.*, 1997).

The peptide hormones, prolactin and insulin, stimulate cell surface receptors which activate cascades of intracellular signalling events. Steroid hormones, by contrast, activate receptors in the cytoplasm (or nucleus) of the cell. This is thought to lead to allosteric changes, dimerisation of the receptor, and nuclear localisation of the activated receptor where it binds to specific recognition sequences in the promoters of target genes. Glucocorticoid receptor binding sites have been identified in the promoters of several milk protein genes (Welte *et al.*, 1993; Malewski, 1998). More recently, CCAAT/enhancer-binding proteins (C/EBPs), which mediate the glucocorticoid response in other cell types and tissues (Ben-Or & Okret, 1993; MacDougald *et al.*, 1994; Williams *et al.*, 1991), have been shown to be important for glucocorticoid signalling in the mammary epithelial cell, and the synergistic activation by lactogenic hormones of milk protein gene expression (Raught *et al.*, 1995).

(v) Transcriptional activation of the milk protein genes -

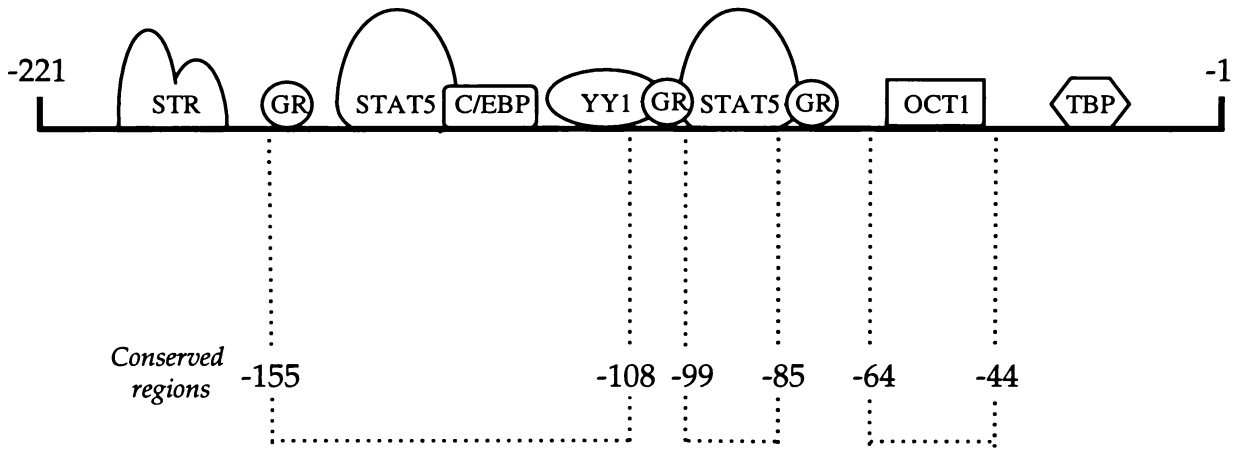
The milk protein genes are expressed in mammary secretory cells by the same basic processes described for other eukaryotic genes. The DNA-dependent RNA polymerase II (which transcribes most eukaryotic genes) initiates the transcription process. Promoters recognised by Polymerase II are characterised by an A/T-rich sequence, containing a TATA box, located approximately 25 base pairs upstream from the transcription start site. The basal transcription factor complex TFII-D (which contains the TATA box-binding protein), and several other initiator proteins are required for assembly of a functional transcription initiation complex. However, eukaryotic genes depend on *trans*-acting (transcription) factors which bind to *cis*-acting elements in the proximal promoter, as well as Polymerase II for their transcription. Additional regulatory elements such as enhancers (located upstream of the proximal promoter, downstream of the genes or within intronic sequences), also play a role. Enhancers modulate promoter activity via interactions with the transcription machinery and transcription factors acting at the proximal promoter. Furthermore, for genes such as the caseins which are part of a large gene locus (αS_1-/β- /αS_2- /κ-casein in the cow and α-/β-/γ-/ε-/κ-casein in the mouse), distal regulatory elements are important for maximal gene expression (Threadgill & Womack, 1990; George *et al.*, 1997; Lee *et al.*, 1989).

Gene expression patterns are controlled by the activity of the transcription factors that regulate transcription. One strategy that is commonly used to control transcription factor activity is the *de novo* synthesis of a transcription factor. Since this implicates the activity of other factors to turn on this gene, this mechanism of control leads to complex patterns of gene expression, and often occurs during determination and differentiation processes. In contrast, rapid changes in gene expression in response to extracellular signals are often mediated by pre-existing factors which are activated by post-translational processes, such as for STAT5 (Darnell *et al.*, 1994; Wang, 1994).

The β-casein proximal promoter has been extensively studied, and contains numerous binding sites for several different positively- and negatively-acting transcription factors (Schmitt-Ney *et al.*, 1991; Lee & Oka, 1992; Altiok & Groner, 1993; 1994; 1998; Raught *et al.*, 1994). A schematic representation of some of the conserved regulatory elements located in the β-casein proximal promoter is shown in **Fig. 5**. Conserved regulatory elements have since been located in the promoter regions of the milk protein genes across several species (Malewski, 1998). In the casein promoters, these include binding sites for STAT5, the glucocorticoid receptor, Yin-Yang 1 (YY1) and the CCAAT/enhancer binding proteins (C/EBPs). In the whey acidic protein promoters, these include binding sites for STAT5, nuclear factor-1 (NF-1) and the glucocorticoid receptor. Although both the WAP and β-casein genes contain STAT5 sites, only WAP gene expression was reduced in STAT5a-deficient mice (Liu *et al.*, 1997). This suggests that additional (and possibly as yet unidentified) transcription factors are important for β-casein gene transcription.

STAT5 was originally identified as mammary gland factor (Wakao *et al.*, 1992), and was later found to be a member of the signal transducer and activator of transcription (STAT) protein family (Wakao *et al.*, 1994). Several isoforms of STAT5 have been identified, and two homologous genes which encode different isoforms of STAT5a and STAT5b have been identified and cloned from the mouse (Liu *et al.*, 1995; Azam *et al.*, 1995; Mui *et al.*, 1995), rat (Kazansky *et al.*, 1995) and human (Hou *et al.*, 1995; Silva *et al.*, 1996). All of the STAT5 isoforms contain the functionally essential tyrosine residue at position 694 (Gouilleux *et al.*, 1994), except for the C-terminally truncated forms which lack this residue and consequently act as dominant negative inhibitors of STAT5 (Mui *et al.*, 1996; Morrigl *et al.*, 1996; Wang *et al.*, 1996).

The consensus DNA motifs specifically recognised by STATs, termed γ-interferon-activated sequences (GAS), consist of the palindromic sequence TTCNNNGAA (Ihle,1996a; Horseman & Yu-Lee, 1994).



adapted from Groner & Gouilleux, (1995).

Figure 5 *The rat β-casein proximal promoter (-221 to -1)*
The sequence-specific single-stranded DNA-binding factor (STR), STAT5, the glucocorticoid receptor (GR), the CCAAT/enhancer-binding proteins (C/EBP), Yin-Yang 1 (YY1), Octamer-1 (OCT1) and the TATA box-binding protein (TBP) are known to participate in protein-DNA and/or protein-protein interactions at the β-casein proximal promoter. The regions between -85 and -99, and -108 and -155 are strongly conserved in the casein genes of several species. Note that activation of gene transcription has been proposed to occur due to a relief of repression by YY1 (see Fig. 6). *(Drawing not to scale).*

The STAT5 binding site was originally identified in the promoter region of the rat β-casein gene (Schmitt-Ney *et al.*, 1991), and was shown to be crucial for prolactin-responsiveness of the β-casein gene *in vitro*. It has also been shown that STAT5-binding sites in the WAP and β-lactoglobulin gene promoters are important for prolactin-responsiveness and maximal gene expression both *in vitro* and *in vivo* (Demmer *et al.*, 1995; Li & Rosen, 1995; Burdon *et al.*, 1994a; 1994b). To date, potential STAT5 binding sites have been identified in the bovine β-, αS_1-, αS_2- and κ-casein genes, the ovine β-lactoglobulin gene and the mouse WAP gene (Groenen & van der Poel, 1994).

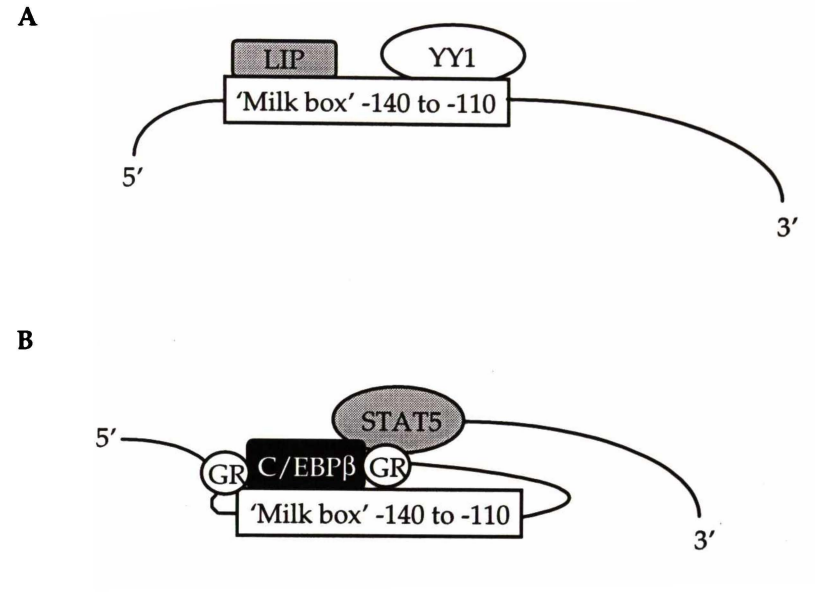
A number of cytokines stimulate the activation of STAT5 and/or transactivate β-casein-reporter genes *in vitro*. In addition to prolactin, these include growth hormone, erythropoietin, granulocyte-macrophage colony stimulating factor (Gouilleux *et al.*, 1995), interleukin-2 (Fujii *et al.*, 1995), interleukin-3 and interleukin-5 (Mui *et al.*, 1995). However, these molecules are unlikely to stimulate milk protein synthesis *in vivo*.

The production of mice in which either the STAT5a and/or STAT5b genes have been inactivated, has provided insight into the roles of these proteins *in vivo*. STAT5a and STAT5b have similar but non-identical tissue distributions, which suggests that these proteins may have distinct roles. In $STAT5a^{-/-}$ mice (Liu *et al.*, 1997) the numbers of alveoli in the mammary gland were significantly reduced, but the most obvious phenotype was the inability of $STAT5a^{-/-}$ mice to lactate due to a failure of the gland to develop and terminally differentiate during pregnancy. Mammary development was also impaired in $STAT5b^{-/-}$ mice (Udy *et al.*, 1997). Milk protein genes were expressed in $STAT5b^{-/-}$ mice, *albeit* at lower levels compared with wildtype littermates (Davey *et al.*, 1996), however there was insufficient milk for the $STAT5b^{-/-}$ females to feed their pups. Of note, $STAT5b^{-/-}$ mice also failed to maintain the normally sexually dimorphic responses mediated by growth hormone (as determined by measuring growth rate and analysing liver gene expression). Mice in which both the STAT5a and STAT5b proteins are absent have

also been produced (Teglund *et al.*, 1998). However, due to the infertility of STAT5a$^{-/-}$5b$^{-/-}$ mice, the effects of the complete absence of both STAT5a and 5b could not be examined in the mammary gland. The results of the previous studies, however, suggest that although these proteins are closely related, STAT5b has at least some distinct physiological functions from STAT5a. It is possible that STAT5a and STAT5b have other essential functions *in vivo*, but that the overlapping functions of other HBP hormones compensate so that these knockout genotypes are not lethal.

In normal mice, the STAT5a and STAT5b proteins are present in the mammary gland throughout the lactation cycle (Liu *et al.*, 1996). However, the activated phosphorylated state of these proteins is highly regulated. Activated STAT5a and STAT5b levels are very low in the virgin animal, but rise sharply after day 14 of pregnancy, and then decrease rapidly during involution. This pattern of activity, and the results from the STAT5 knockout mice, suggest that the activation of STAT5a and STAT5b is important for terminal differentiation of the mammary gland before lactation commences, and the initiation of milk protein gene expression.

The widely expressed zinc finger protein YY1 (Shi *et al.*, 1991), by contrast, has been shown to act as a transcriptional repressor in the β-casein gene promoter. The presence of consensus sequences for this factor in the promoters of a number of the milk protein genes (Malewski, 1998), suggests a general role for YY1 in the regulation of milk protein gene expression. YY1 binds to a repressor region located between positions -150 and -100 of the rat β-casein promoter, to suppress transcription in the uninduced state. Repression by YY1 is counteracted by lactogenic hormone treatment, and the subsequent binding of STAT5 to the promoter (Meier & Groner, 1994; Raught *et al.*, 1994). It is thought that direct interactions between STAT5 and the glucocorticoid receptor, and C/EBPs and the glucocorticoid receptor also facilitate the displacement of YY1, which leads to the transcriptional activation of the β-casein gene (see **Fig. 6**) (Rosen *et al.*, 1998).



adapted from Rosen et al., (1998)

Figure 6 *Hormonal activation of casein gene expression.*

(A) A simplified model of the rat β-casein promoter containing the 'milk box' region (-140 to -110) and its interaction with the transcription factors LIP and YY1, which repress transcription in the uninduced state. (B) Activation of the β-casein promoter by glucocorticoids and prolactin results in a glucocorticoid-dependent switch in the C/EBPβ isoforms from LIP to LAP, and the induction of C/EBPα. Prolactin activation of STAT5 results in protein-DNA as well as protein-protein interactions between STAT5 and the glucocorticoid receptor (GR), and possibly C/EBP and STAT5, which facilitates the displacement of YY1 and results in gene activation. C/EBP, CCAAT/enhancer-binding protein; LAP, liver-enriched activating protein; LIP, liver-enriched inhibiting protein; STAT, signal transducer and activator of transcription; YY1, Yin-Yang 1.

C/EBP proteins are known to be mediators of the glucocorticoid response in many cells and tissues (Ben-Or & Okret, 1993; MacDougald *et al.*, 1994; Williams *et al.*, 1991), and are thought to play a similar role in the mammary gland. Highly conserved C/EBP binding sites are found in the rat (mouse, rabbit and bovine) β-casein promoter at around position -135 and between positions -220 and -156 (Raught *et al.*, 1995). These sites are essential for maximal β-casein gene expression in response to lactogenic hormones (Doppler *et al.*, 1995). The C/EBP proteins (C/EBPα, -β, -δ and -γ) are sequence-specific transcription factors and are characterised by conserved DNA-binding domains. Consequently, all of the C/EBP isoforms are capable of interacting with the C/EBP consensus sequence as homo- and heterodimers. In particular, the levels of C/EBPα and C/EBPβ change dramatically throughout mammary gland development (Raught *et al.*, 1995). The levels of C/EBPα are very low in the non-lactating gland, but are dramatically increased during lactation. In contrast, C/EBPβ predominates in the non-lactating gland. The levels of C/EBPβ gradually increase during pregnancy, and then dramatically decrease at parturition. There are three forms of C/EBPβ, known as LAP1 (liver-enriched activating protein 1), LAP2 and LIP (liver-enriched inhibiting protein). Since LIP does not possess a transactivating domain, it can form heterodimers with other members of the C/EBP family and suppress their transcriptional activity. Thus, the LAP/LIP ratio is thought to be an important indicator of C/EBP activity. Since glucocorticoids decrease the levels of LIP, these hormones may mediate their effects on milk protein gene expression, in part, by influencing the LAP/LIP ratio and C/EBPβ activity.

More recently, studies using C/EBP-deficient mice have confirmed that the C/EBP proteins are important regulators of gene expression in many tissues and cell types, including the mammary gland. Although C/EBPα$^{-/-}$ mice die shortly after birth and C/EBPβ$^{-/-}$ mice have reproductive problems, mammary and ovarian transplantation and primary cell culture experiments

have revealed that C/EBPβ, but not C/EBPα, is essential for ductal and lobulo-alveolar development, functional differentiation and milk protein gene expression in the mouse mammary gland (Robinson *et al.*, 1998; Seagroves *et al.*, 1998).

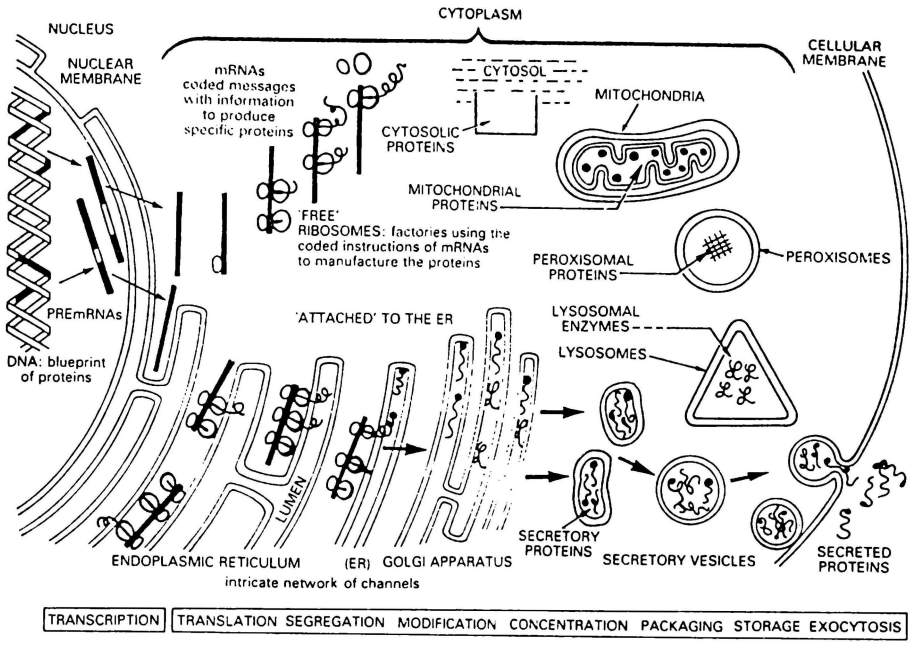
Several other negatively-acting regulators of milk protein gene expression have also been identified. For instance, a sequence-specific single-stranded DNA-binding factor has been found to bind to the β-casein promoter (between positions -221 and -170) and compete with an activating factor, therefore having a negative regulatory role (Altiok & Groner, 1993). In addition, a pregnancy-specific mammary nuclear factor has been shown to bind to the β-casein promoter. This factor is regulated by progesterone and is thought to act as a transcriptional repressor (Lee & Oka, 1992), consistent with the negative effects of progesterone on milk protein gene expression in mouse mammary epithelial cells in culture (Terada *et al.*, 1988).

Much work has focused on examining the process by which mammary epithelial cells integrate multiple hormonal and ECM-derived signals to regulate milk protein gene expression. It has been established that the β-casein proximal promoter contains highly conserved consensus sequences for numerous *trans*-acting factors. It is thought that composite response elements may provide a possible mechanism for distinct signalling pathways to converge at the level of transcription. Some evidence for this comes from the results of recent studies which have shown that STAT5 can interact with the glucocorticoid and insulin receptors (Cella *et al.*, 1998; Lechner *et al.*, 1997a; 1997b; Stöcklin *et al.*, 1996; 1997; Chen *et al.*, 1997; Sawka-Verhelle *et al.*, 1997), as well as C/EBPs (Rosen *et al.*, 1998). In a broader sense, the transcription factor families of NF-κB/Rel, STAT and C/EBP have been shown to interact with steroid receptors (for a review see Gottlicher *et al.*, 1998).

The exact molecular mechanisms by which the ECM modulates expression of the tissue-specific milk protein genes are poorly understood. Some progress has been made with the identification of a unique 160 bp ECM-dependent enhancer (BCE-1), approximately 1.6 kb upstream of the bovine β-casein transcription start site, which is thought to mediate changes in gene expression in response to the ECM (Schmidhauser *et al.*, 1990; 1992). More recently, it has been suggested that ECM-derived signals may induce complex interactions between the transcription factors working at this site, the basal transcription machinery and chromosomal structure (Myers *et al.*, 1998). Both BCE-1 and the β-lactoglobulin promoter contain STAT5 binding sites, and mutations within the STAT5 binding sites of the latter have been shown to abolish ECM-dependent activity, suggesting that STAT5 may be a target for regulation by the ECM (Streuli *et al.*, 1995a; 1998). It has also been suggested that protein-tyrosine phosphatases may be targets for regulation by the ECM (Edwards *et al.*, 1998). These results provide possible mechanisms for distinct signalling pathways, activated in response to either hormonal or ECM-derived signals, to converge on a single pathway to regulate transcription of the milk protein genes. However, alternate ECM-dependent pathways may also operate.

(vi) Post-transcriptional regulation of milk protein gene expression -

The focus of this review has been on the hormonal regulation of milk protein gene expression at the transcriptional level. However, it should be pointed out that in addition to transcriptional activation of the milk protein genes, messenger RNA/transcript stability, protein synthesis, protein degradation and secretion are all considered to be important levels of control within the mammary epithelial cell. For instance, mammary cells require numerous precursors for milk production, including glucose, fatty acids, amino acids and minerals, which are absorbed from the blood across the basolateral membrane (Larson, 1985).



adapted from Mepham, (1987).

Figure 7 *Schematic representation of the intracellular transport of proteins in mammary cells*

For protein synthesis, the availability of these precursors is an important level of control and is dependent on the diet of the lactating animal, the utilisation of the precursors by extra-mammary tissues, the rate of uptake by the gland, and partitioning between the individual metabolic pathways of the secretory cell.

Protein synthesis occurs in the rough endoplasmic reticulum of the cell from amino acid precursors derived from the blood, and then proteins are subject to post-translational modification prior to secretion through the Golgi in secretory vesicles. In particular, the most abundant milk proteins, caseins, are phosphorylated and glycosylated in the Golgi, which is also the site of calcium-dependent micelle formation prior to secretion. Protein secretion is believed to occur via a mechanism described by Blobel *et al.*, (1979) as the 'signal hypothesis'. A schematic representation of the process of intracellular transport of proteins in mammary cells is illustrated in **Fig. 7**.

Although the process of secretion is not completely understood, the release of secretory proteins is thought to occur via two main process, constitutive and regulated secretion (for a review see Burgoyne *et al.*, 1998).

In summary, the overall control of milk protein gene expression is complex, and is regulated by multiple intracellular signalling pathways stimulated by the lactogenic hormones and the extracellular matrix. Although a number of factors are known to be involved in linking extracellular stimuli with downstream responses, the exact molecular mechanisms which integrate all of the extracellular signals governing the tissue-specific expression of milk protein genes remain to be established.

OBJECTIVES OF THIS RESEARCH

Prolactin plays a pivotal role during the initiation of lactation, and in conjunction with other signals stimulates the development and differentiation of the mammary gland. These responses to prolactin are mediated through the activation of specific genes, to alter the overall pattern of gene expression. A number of factors are known to be involved in this process, and a comprehensive picture of the intracellular signalling pathways for prolactin is emerging.

Prolactin stimulates multiple intracellular signalling pathways in a variety of tissues and cell types, by processes which involve sets of common signalling molecules. In mammary epithelial cells, specific biological outcomes are determined by the combination of hormonally- and developmentally-regulated transcription factors, participating in protein-DNA and protein-protein interactions at the proximal promoters of target genes. Certain signalling molecules and *trans*-acting factors have been implicated in this process, however, the exact molecular mechanisms controlling the integration of multiple extracellular stimuli, and their

subsequent effects on milk protein gene expression are not yet clearly defined.

While rapid responses to prolactin are mediated via pre-existing molecules, the *de novo* synthesis of other controlling proteins may be required to mediate the pleiotropic effects of prolactin, and to achieve the complex pattern of gene expression in the lactating gland. This project explores the *de novo* synthesis of proteins in response to prolactin in mouse mammary epithelial cells, with the aim of identifying proteins which are involved in mediating the lactogenic effects of prolactin. The identification of these proteins may provide clues to the underlying molecular events regulating milk protein gene expression, and will improve our knowledge in this area.

Two main approaches will be taken in an attempt to achieve this goal. First, the results of proteome analyses using large-format two-dimensional electrophoresis will be used to identify lactation-associated and prolactin-responsive proteins. This is an extremely powerful method of detecting changes in cellular protein synthesis, and a number of hormonally-responsive mammary proteins have been detected using this approach. These proteins will be assessed as potential regulators of lactation in mammary cells. Second, this project will investigate the role of *de novo* protein synthesis during lactogenic signalling in mouse mammary epithelial cells. Thus, this project represents a broad effort to improve our knowledge of the molecular mechanisms which regulate milk protein gene expression.

Chapter Two - Materials and Methods

General methods

Standard molecular biology methods including phenol/chloroform extraction and precipitation of nucleic acids, electrophoresis, hybridisations, Northern blotting and restriction digestions were performed essentially as described by Sambrook *et al.* (1989) and in Ausubel *et al.* (1987). Common lab reagents were obtained from BDH Chemicals NZ, Ltd. (Palmerston North) and Sigma Chemical Co. (MO, USA). Restriction enzymes were obtained from Boehringer Mannheim NZ Ltd., (Auckland). Radiolabelled nucleotides were from Amersham Life Science (Buckinghamshire, UK). *Milli Q* filtered water (Millipore Corp., MA, USA) was used for all molecular biology and cell culture experiments. All cell culture experiments were carried out under aseptic conditions using sterile solutions and equipment. Diethyl pyrocarbonate (DEPC)-treated water and solutions were used for all experiments with RNA.

Cell Culture

Standard reagents and cell culture solutions are listed in **Table 2.**

(i) Preparation of collagen gels -

Collagen preparation and titration were carried out essentially as described by Richards *et al.* (1983). Tendon fibres were dissected from rat tails and sterilised in 70% ethanol for 30 min. The fibres were added to 0.017 M acetic acid at 8 g/L and the suspension was stirred for 4 d at 4°C to extract the collagen. The collagen solution was centrifuged at 10,000 x *g* for 1 h at 4°C. The top third of the solution was decanted off and the remainder diluted to a concentration of 0.5-1.0 mg/mL total protein with 0.017 M acetic acid and stored at 4°C.

Table 2 *Cell Culture Reagents*

Anisomycin	reconstituted in DMSO (Sigma Cell Culture, MO, USA)
Antibiotic solution	10,000 U/mL penicillin G and 10 mg/mL streptomycin
Antibiotic/antimycotic solution	10,000 U/mL penicillin G, 10 mg/mL streptomycin and 25 µg/mL amphoterycin B
Collagenase	Type V from *Clostridium histolyticum* (Sigma Cell Culture, MO, USA)
Crystal violet	0.04% crystal violet/1 M citric acid
Cycloheximide	reconstituted in water (Sigma Cell Culture, MO, USA)
5,6-Dichlorobenzimidazole riboside (DRB)	reconstituted in water (Sigma Cell Culture, MO, USA)
Dispase	Neutral protease from *Bacillus polymyxa*, Grade 2 (Boehringer Mannheim NZ Ltd., Auckland)
DMEM	Dulbecco's Modified Eagle's medium, high glucose (GIBCO BRL Life Technologies, Inc., NY, USA)
DMEM:F12	Dulbecco's Modified Eagle's medium:F12 nutrient mixture (GIBCO BRL Life Technologies, Inc., NY, USA)
DMEM, met$^-$ cyst$^-$	Dulbecco's Modified Eagle's medium, methionine and cysteine depleted (Sigma Cell Culture, MO, USA)
Epidermal growth factor	from mouse maxillary glands (Boehringer Mannheim NZ Ltd., Auckland)
Foetal Bovine Serum	not heat inactivated (Gibco BRL Life Technologies, Inc., NY, USA)
Hydrocortisone	Kendall's compound F, cortisol, approximately 98% (Sigma Cell Culture, MO, USA)
Insulin	from bovine pancreas, 24.4 IU/mg (Sigma Cell Culture, MO, USA)
Passaging solution for COMMA-D cells	2.4 mg/mL dispase/10 mM glucose/100 mM sodium chloride/3 mM potassium chloride/1 mM Na_2HPO_4/30 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid]
Prolactin	from ovine pituitary, 31 IU/mg (Sigma Cell Culture, MO, USA)

An empirically derived method was used to titrate the collagen in order to produce consistent gels from each batch of collagen. Six different neutralising solutions consisting of 0.34 M sodium hydroxide and 10x concentrated Waymouth's medium in the ratios of 1.0:2.0, 1.0:1.9, 1.0:1.8, 1.0:1.7, 1.0:1.6 and 1.0:1.5 (v/v) were prepared in an ice bath. Aliquots of the collagen solution (4.0 mL) were titrated to pH 7.4 using each of the neutralising solutions to initiate crosslinking of collagen fibrils. The neutralising solution giving a gel that remained intact when picked up with blunt forceps and gelled within 5 min at room temperature (RT) after being neutralised (but not after 10 min on ice) was chosen. Collagen stock solution was neutralised to pH 7.4 in an ice bath using the selected neutralising solution and aliquoted at 190 $\mu L/cm^2$ (for collagen gels) or 15 $\mu L/cm^2$ (for thin coating) of growth surface. The collagen was spread evenly over the growth surface and incubated for 30 min at RT to allow gelling. Cells were plated immediately thereafter.

(ii) Matrigel® thin coating method -

Matrigel® basement membrane matrix (obtained from Collaborative Biomedical Products, Becton-Dickinson Labware, MA, USA) is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumour rich in extracellular matrix proteins. It contains laminin, collagen IV and heparan sulphate proteoglycans, providing a complex protein layer on top of which cells can grow. *Matrigel®* basement membrane stored at -70°C was thawed at 4°C immediately before use, mixed to homogeneity and aliquoted at 9 $\mu L/cm^2$ of growth surface in an ice bath. The matrix was spread evenly over the cooled growth surface, incubated for 30 min at RT to allow gelling and the cells plated immediately thereafter.

(iii) COMMA-D cell culture -

COMMA-D cells were a gift from Dr Dan Medina (Baylor College of Medicine, TX, USA). All experiments were carried out using cultures at passages 15-29 since initial isolation (Danielson *et al.*, 1984). Cells were

cultured in growth medium containing 0.1 mg/mL streptomycin, 100 U/mL Penicillin G, 0.25 µg/mL amphotericin, 5% (v/v) foetal bovine serum, 6 µg/mL insulin and 5 ng/mL epidermal growth factor (EGF) in Dulbecco's modified Eagle's medium:F12 nutrient mixture (DMEM:F12). Cells were passaged at 1/6 dilution when confluent by incubating the cells in a minimal volume of passaging solution (**Table 2**) for 30 min at 37°C. For all experiments COMMA-D cells were plated at 6 x 10^5cells/cm^2 onto plastic, collagen- or *Matrigel*®- coated plates and cultured in growth medium for 2 d. On day 2 of culture, serum and EGF were withdrawn and cells were cultured in DMEM:F12 medium containing 6 µg/mL insulin and 3 µg/mL hydrocortisone. This medium was supplemented with 5 µg/mL prolactin from day 3 in culture to induce expression of milk proteins.

(iv) Dissociation and isolation of primary mouse mammary epithelial cells -

CF1 mice were obtained from the Small Animal Colony (AgResearch, Ruakura Research Centre, Hamilton, NZ) and sacrificed by CO_2 euthanasia. The fourth and fifth mammary glands were recovered, pooled and finely minced with scissors. The tissue was dissociated on an orbital shaker for 50 min at 37°C in Dulbecco's modified Eagle's medium (DMEM) which contained 0.1 mg/mL streptomycin, 100 U/mL Penicillin G, 0.25 µg/mL amphotericin, 5% (v/v) foetal bovine serum, 5 µg/mL insulin and 3 µg/mL hydrocortisone. This basal medium was supplemented with 1000 U/mL collagenase to facilitate tissue dissociation. Undissociated tissue was removed by filtering the cell suspension through a coarse gauze filter (approximately 300 µm diameter). Cell clusters were pelleted by centrifugation at 150 x *g* for 5 min and the cell pellet was washed a further two times in basal medium. The cells were pelleted between washes by centrifugation at 100 x *g* for 3 min (first wash) and 50 x *g* for 3 min (second wash) to enrich for clumps of epithelial cells (Neville *et al.*, 1991).

For proteome analysis, cells were isolated from day 10 pregnant or day 7 lactating mice and plated at 3 x 10^5 cells/cm^2 onto plastic in basal medium immediately after isolation and metabolically labelled as described below. For other experiments, cells isolated from day 14-15 pregnant CF1 or *STAT5b*$^{-/-}$ mice were plated onto plastic, collagen- or *Matrigel*®- coated plates at 3 x 10^5 cells/cm^2 in basal medium. These cells were cultured for 24 h and then in serum-free DMEM containing 0.1 mg/mL streptomycin, 100 U/mL Penicillin G, 0.25 µg/mL amphotericin, 5 µg/mL insulin and 3 µg/mL hydrocortisone. On day 3 in culture, the medium was supplemented with 5 µg/mL prolactin to induce expression of milk proteins. A subcutaneous delivery of progesterone (about 4 mg; s.c. implantation on day 5 to 7 of pregnancy of a 5 x 1.5 x 1.5-mm segment of a controlled intravaginal drug release device; CIDR, InterAg, Hamilton, NZ) was administered to *STAT5b*$^{-/-}$ females to maintain pregnancy (Udy *et al.*, 1997). *STAT5b*$^{-/-}$ mice were kindly provided by Dr. Helen Davey (Dairy Science, AgResearch, Hamilton, NZ).

Proteome Analysis

(i) Metabolic labelling of cultured cells -

Cells were metabolically labelled with a mixture of [^{35}S]methionine and [^{35}S]cysteine as follows. Primary cells isolated from day 10 pregnant or day 7 lactating mice were labelled 15 min after plating and COMMA-D cells were cultured as described above and labelled three days after prolactin treatment. Prior to labelling, cells were incubated in methionine- and cysteine-depleted DMEM for 15 min. Cells were then incubated for 2 h at 37°C in the same medium containing 0.2 mCi/mL [^{35}S]methionine, 5 µg/mL insulin (for primary cultures or 6 µg/mL for COMMA-D cultures), 3 µg/mL hydrocortisone and 5 µg/mL prolactin. Control cultures contained no prolactin. Following the incubation, cells were recovered from the culture surface by vigorous trituration with ice cold phosphate buffered saline (PBS, pH 7.4). Cells were recovered from the suspension by centrifugation at 150 x *g* for 3 min.

(ii) Subcellular fractionation -

Subcellular fractionation was performed essentially as previously described (Fleischer & Kervina, 1974). Cells were resuspended in low salt buffer (**Table 3**) containing 0.5% (v/v) Nonidet-P40 (NP-40) and incubated for 10 min in an ice bath. The solution was then passed 10 times through a 20 gauge needle and syringe and a portion of the cell lysate was resuspended in sample lysis buffer containing 9.5 M urea, 2% (v/v) NP-40, 1.6% (w/v) ampholytes pH range 5-7, 0.4% (w/v) ampholytes pH range 3.5-10 and 5% (v/v) 2-mercaptoethanol (O'Farrell, 1975). The remaining cell lysate was centrifuged at 150 x *g* for 5 min to pellet the nuclei and the supernatant was centrifuged at 100,000 x *g* for 60 min at 4°C. This high speed pellet was resuspended in sample lysis buffer (membrane/organelle fraction). The high speed supernatant (cytosolic fraction) was dialysed for 5 h at 4°C against water, lyophilised and resuspended in sample lysis buffer.

High salt extracts of nuclei were prepared as previously described by Dignam (1983) with the following modifications. The crude nuclei pellet was resuspended in 1 mL low salt buffer containing 0.5% (v/v) NP-40, the nuclei centrifuged at 1500 x *g* for 5 min and resuspended a second time in 1 mL low salt buffer, this time in the absence of NP-40. An equal volume of high salt buffer (**Table 3**) was added and the nuclei were extracted in 0.3 M potassium chloride. Extracts were dialysed overnight against water at 4°C, lyophilised and resuspended in sample lysis buffer.

(iii) Trichloroacetic acid assay to determine radioactivity incorporated into proteins -

Equal volumes of samples to be assayed (2 µL) were aliquoted onto the centre of filter paper squares (approximately 1 cm^2). Each sample was analysed in duplicate. The filters were dried for at least 1 h at RT and then incubated in 10% (w/v) trichloroacetic acid (TCA) containing 0.1% (w/v) methionine for 1 h at 4°C. The filters were then washed in 5% (w/v) TCA for 30 min, washed three times for 5 min in 95% (v/v) ethanol and dried at RT.

Table 3 *Standard Solutions*

Dialysis buffer	20 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid], pH 7.9/ 1.5 mM magnesium chloride/100 mM potassium chloride/0.2 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/ 1 mM sodium vanadate/ 20% (v/v) glycerol
10 x EMSA sample loading buffer	250 mM Tris, pH 7.5/0.2% bromophenol blue/40% (v/v) glycerol
Gel loading dye	0.25% bromophenol blue/0.25% xylene cyanol/1 mM EDTA/ 50% (v/v) glycerol
High salt buffer	20 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid], pH 7.9/ 1.5 mM magnesium chloride/600 mM potassium chloride/0.5 mM DTT/0.2 mM phenylmethylsulfonyl fluoride/ 1 mM sodium vanadate/25% (v/v) glycerol
10 x Klenow buffer	500 mM Tris, pH 7.5/100 mM magnesium chloride/10 mM DTT/ 0.5 mg/mL bovine serum albumin
5x labelling mix	250 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid], pH 6.6/25 mM magnesium chloride/50 mM β-mercaptoethanol/2 mg/mL bovine serum albumin/1 mM dATP/ 1 mM dGTP/1 mM dTTP
Low salt buffer	10 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid], pH 7.9/1.5 mM magnesium chloride/10 mM potassium chloride/0.5 mM DTT/0.2 mM phenylmethylsulfonyl fluoride/ 1 mM sodium vanadate
10xMOPS	0.2 M 3-(*N*-morpholine)propanesulphonic acid, pH 7.0/80 mM sodium acetate/10 mM EDTA
10xSSC	1.5 mM sodium chloride/150 mM sodium citrate
TBE buffer	45 mM Tris/45 mM boric acid/1 mM EDTA
TE	10 mM Tris, pH 7.6/1 mM EDTA

Filters were transferred to 2.5 mL scintillant (Beckman Ready Safe Scintillation Cocktail, Beckman Instruments Inc., CA, USA) in scintillation vials and counted for 60 seconds in a *Wallac 1409 Liquid Scintillation Counter* (Wallac, Finland).

(iv) One-dimensional and two-dimensional polyacrylamide-SDS electrophoresis -

Acrylamide, ammonium persulphate (APS), bromophenol blue, Coomassie brilliant blue G-250, glycine, ion exchange resin (molecular biology grade, AGR501-X8 D), *N, N'*-methylene-bis-acrylamide, molecular weight markers (range 14,400-97,400 Daltons), *N, N, N', N'*- tetra-methylenediamine (TEMED), polyoxyethylene sorbitan monolaurate (Tween-20) and urea were all electrophoresis purity and from Bio-Rad laboratories, (CA, USA). Tris and sodium salicylate were from Boehringer Mannheim NZ Ltd., (Auckland). Ampholytes (pH ranges 3.5-10, 5-7 and 7-9) were from Pharmacia LKB, (Sweden).

Discontinuous one-dimensional polyacrylamide-SDS gel electrophoresis was performed essentially as described by Laemmli (1970). Polyacrylamide gels (160 x 200 x 1.5 or 0.75mm) were prepared and electrophoresis was carried out in a Bio-Rad *Protean™ II* Slab Cell apparatus. Separating gels contained between 9-12% (w/v) acrylamide/0.24-0.32% (w/v) *N,N'*-methylene-bis-acrylamide/0.1% SDS/0.375 M Tris, pH 8.8 and stacking gels contained 4% (w/v) acrylamide/0.1% (w/v) *N, N'*-methylene-bis-acrylamide/0.1% SDS/0.05 M Tris, pH 6.9.

Two-dimensional polyacrylamide-SDS electrophoresis separates proteins according to charge in the first dimension and molecular weight in the second dimension. The procedure was carried out essentially as described by O'Farrell, (1975) except that large-format gels (35 x 25 x 0.075 cm) were used as described by Young *et al.*, (1983). Briefly, samples of equivalent counts were loaded to pre-focused first dimension gels (370 x 3 mm i.d.) containing 1% (w/v) ampholytes pH range 3.5-10 and 1% (w/v) ampholytes

pH range 5-7. First dimension gels were equilibrated in buffer containing 10% (w/v) glycerol/ 5% (v/v) 2-mercaptoethanol/ 2% (w/v) SDS/ 0.0625 M Tris for 15 min and attached to 12% (w/v) acrylamide-SDS separating gels. A constant current of 24.5 mA/gel was applied from the anode until the dye fronts were 1-2 cm from the bottom of the gels (18-21 h). Gels were fixed in a solution containing 50% (v/v) methanol/12% (v/v) acetic acid or stained in a solution containing 0.2% (w/v) Coomassie brilliant blue-G250/40% (v/v) methanol/10% (v/v) acetic acid. Dried gels were exposed to *X-Omat AR* film (Eastman Kodak Co., NY, USA) in the *Ready Pack* envelopes in which the film is supplied by resealing them with tape. The film was exposed for 60 x 10^6 cpm.days. For some of the gels the radiolabelled proteins were detected by fluorography. In this case, gels were soaked in 1 M sodium salicylate for 1 h (Chamberlain, 1979), then rinsed briefly in water immediately before drying. Dried gels were exposed to pre-flashed *X-Omat AR* film (15 x 10^6 cpm.days). Film was pre-exposed to a background absorbance increment of 0.1-0.15 at 540 nm (Laskey and Mills, 1975).

(v) HPLC and amino acid sequencing -

Gels were stained with Coomassie blue, destained and the protein spot was cut out. Proteolysis was then carried out within the gel using *Endoproteinase Lys C* (Sequencing grade, Promega Corp., WI, USA). The acrylamide gel piece containing the protein was washed twice for 15 min in 50% (v/v) methanol, twice for 15 min in water and three times for 10 min in 50 mM Tris, pH 7.9/1 mM EDTA. The gel was crushed in a microfuge tube using a pestle in a minimal volume of buffer. *Lys C* was added to a final concentration of 1.1 mg/mL and the mixture was vortexed at 37°C overnight. The liquid was separated from the gel using a microfilter (Chuang and Blattner, 1994). The gel pieces were washed in the filter with a half volume of fresh buffer and the filtrates combined. The filtrate was lyophilised to approximately 0.05 mL. Proteolytic digests of protein were acidified by adding 10 µL 10% (v/v) trifluoroacetic acid and subjected to HPLC using a 250 x 2 mm i.d. C-18 reverse phase column with a stationary

phase solvent of 0.1% (v/v) trifluoroacetic acid. Peptides were eluted using a gradient of 0 to 50% (v/v) solvent B, where solvent B contains 80% (v/v) acetonitrile and 0.08% (v/v) trifluoroacetic acid. Selected peptides were subjected to amino acid sequencing using a gas phase sequenator (*model 470A*, Applied Biosystems). Amino acid sequencing was performed by Catriona Knight (University of Auckland, New Zealand).

Western analysis

Following electrophoresis, proteins were transferred to 0.45 micron supported nitrocellulose or Polyscreen® PVDF transfer membrane (NEN® Research Products, MA, USA) in 10 mM CAPS/10% (v/v) methanol, pH 11 at 70 V (constant voltage) for 2 h. The membranes were stained with 0.1% (w/v) Ponceau S/0.1% acetic acid (for nitrocellulose membranes) or 0.025% (w/v) Coomassie blue/40% (v/v) methanol/5% (v/v) acetic acid (for PVDF membranes). Following staining, the membranes were incubated in blocking solution (**Table 4**), washed three times for 5 min in 0.19 M sodium chloride/0.1 M Tris, pH 7.4 (TBS) containing 0.1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween-20, then probed for 2 h with primary antibodies (see **Appendix 1**). The membrane was washed again three times for 5 min in TBS/BSA/Tween-20 and then incubated for 2 h with secondary antibodies (see **Appendix 1**), conjugated to horse radish peroxidase (see **Table 4**). The membranes were washed four times for 5 min in TBS/BSA/Tween-20 before detection of the signal using either 3,3'-diaminobenzene tetrahydrochloride (DAB) or enhanced chemiluminescence (*ECL* detection kit, Amersham Life Sciences, Buckinghamshire, UK) according to manufacturer's instructions.

Table 4 *Western analysis*

Blocking Solution	*Primary antibodies*	*Secondary Antibodies*	*Detection*
4% (w/v) bovine serum albumin in TBS[1]	1/1000 dilution* of anti- **mouse milk** antibodies raised in rabbit[2]	1/10,000 dilution* of rabbit immunoglobulins (raised in goat) from Sigma Chemical Co. (MO, USA)	DAB[3] or *ECL*[4]
4% (w/v) non-fat milk powder in TBS[1]	1/2000 dilution* of anti- **Stat5a** or **Stat5b** antibodies from Santa Cruz Biotechnology, Inc., CA, USA	1/10,000 dilution* of rabbit immunoglobulins (raised in goat) from Sigma Chemical Co. (MO, USA)	*ECL*[4]
4% (w/v) non-fat milk powder in TBS[1]	1/500 dilution* of anti-**grp78** antibodies raised in goat from Santa Cruz Biotechnology, Inc., CA, USA	1/20,000 dilution* of goat immunoglobulins (raised in rabbit) from Sigma Chemical Co. (MO, USA)	*ECL*[4]

1 TBS contains 0.19 M sodium chloride/0.1 M Tris, pH 7.4

2 Anti- mouse milk antibodies were kindly provided by Dr. Brydon Bennett, University of Otago, Dunedin, NZ (now at Signal Pharmaceuticals, Inc., CA, USA)

3 0.5 mg/mL 3,3'-diaminobenzene tetrahydrochloride (DAB)/0.03% hydrogen peroxide/0.19 M sodium chloride/0.1 M Tris, pH 7.4

4 *ECL* detection system (Amersham Life Science, Buckinghamshire, UK) used according to manufacturer's instructions

* All antibodies were diluted in TBS containing 0.1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween-20

Northern analysis

(i) Isolation of RNA -

Mammary tissue (fourth and fifth contralateral glands) was isolated from mice at various stages of the lactation cycle and frozen immediately for RNA isolation. Total RNA was extracted from the tissue using either the guanidinium-acid-phenol-chloroform method (Chomczynski & Sacchi, 1987) or TRIZOL® reagent (total RNA isolation reagent, Gibco BRL, Life Technologies, Inc., NY, USA). Mammary tissue was homogenised with an *Ultra-Turrax T25* machine (Janke & Kunkel IKA®-Labortechnik) in guanidinium or TRIZOL® solution. For cells in culture, RNA was isolated following direct addition of guanidinium or TRIZOL® solution to the attached cells.

Following extraction with either guanidinium or TRIZOL®, RNA in the aqueous phase was precipitated with an equal volume of isopropyl alcohol (AnalaR® grade, BDH Laboratory Supplies, Poole, England) for at least 2 h at -20°C. The RNA was collected by centrifugation at 10,000 x *g* for 10 min at 4°C and washed with 80% (v/v) ethanol (AnalaR® grade, BDH Laboratory Supplies, Poole, England) to remove traces of guanidinium or TRIZOL®. The RNA was resuspended in 100 µL DEPC-treated water and stored at -80°C. The concentration of RNA in each sample was estimated using a UV spectrophotometer (*Shimadzu UV-160A* visible recording spectrophotometer, Kyoto, Japan) assuming that a 40 µg/mL solution has an absorbance of 1 at 260 nm (Sambrook *et al.*, 1989).

(ii) RNA electrophoresis and Northern blotting -

Equivalent amounts of RNA (5 µg) were lyophilised and resuspended in RNA loading buffer containing 10 µL deionised formamide, 3.5 µL formaldehyde, 1 µL 10X MOPS and 2 µL gel loading dye (**Table 3**) in a total volume of 20 µL. RNA samples were heat denatured at 65°C for 10 min and incubated immediately on ice before electrophoresis in a 1.2% (w/v) agarose/1.8% (v/v) formaldehyde gel at 5 V/cm. Following electrophoresis, the

gel was washed in 3 gel volumes of 10x SSC buffer (**Table 3**) for 30 min to remove the formaldehyde from the gel and blotted onto Hybond-N nylon membrane (Amersham Life Science, Buckinghamshire, UK) by capillary transfer. RNA was transferred from the gel for approximately 16 h using 10x SSC as a transfer buffer. Transferred RNA was then cross-linked to the membrane using a *UV Stratalinker® 1800* (Stratagene Cloning Systems, CA, USA).

(iii) Northern hybridisation -

Cloned cDNAs were labelled using a random priming method as follows. Firstly, cDNAs were recovered from plasmids by restriction digestion and electrophoresis in agarose gels. Excised bands were frozen at -70°C and the DNA was separated from the gel using a microfilter (Chuang & Blattner, 1994). An estimated 25 ng of the resulting cDNA was mixed with 3 µg random hexamers in a final volume of 12.5 µL TE (**Table 3**), heated in a boiling water bath for 10 min then cooled immediately on ice. 5 µL of 5x labelling mix (**Table 3**), 25 µCi [α-^{32}P]dCTP (3000 Ci/mmol) and 4 U Klenow enzyme (Boehringer Mannheim NZ, Ltd., Auckland) were added and the mixture was incubated at 37°C for 1-4 h. Alternatively, gel purified cDNA was labelled using a *Rediprime™* kit (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions. Grp78 cDNA was kindly provided by Dr. Hugh Pelham, MRC Laboratory of Molecular Biology, Cambridge, UK (Munro & Pelham, 1986). PDI cDNA was kindly provided by Dr. Jerome Demmer, Dairy Science, AgResearch, Hamilton, NZ (Demmer *et al.*, 1997).

Northern hybridisations were carried out at 65°C overnight in 5 mL hybridisation solution containing 500 mM $NaHPO_4$/1 mM EDTA/7% SDS. Blots were pre-hybridised in this solution for at least 1 h before addition of the denatured probe. Probes were denatured by the addition of 0.2 volumes 4 M sodium hydroxide and incubation at 65°C for 5 min, or by heating in a boiling water bath for 10 min. Probes were then added directly to the pre-hybridisation buffer. After hybridisation, blots were washed twice in 2x SSC/0.1% SDS for 15

min each wash to reduce background signal. Hybridised signal was visualised by exposing blots to *X-Omat AR* film using intensifying screens at -70°C.

Mobility Shift Assays

(i) Preparation of nuclear extracts -

COMMA-D cells

High salt extracts of nuclei were prepared as previously described by Dignam (1983) with the following modifications. Cultured COMMA-D cells (approximately 50-100 x 10^6 cells per condition) were scraped from the cell culture surface into ice cold PBS and the cells centrifuged at 400 x *g* for 2 min at RT. Cells were then sheared in 1 mL low salt buffer (**Table 3**) containing 0.5% NP-40 using a Potter-Elvehjem homogeniser with a teflon pestle. The sheared cells were centrifuged at 1500 x *g* at 4°C and the nuclei washed two times in 1 packed nuclei volume of low salt buffer. The nuclear pellet was resuspended in 1 volume of low salt buffer, to which an equal volume of high salt buffer (**Table 3**) was mixed, resulting in a final concentration of 0.3 M potassium chloride. Nuclear proteins were extracted for 30 min at 4°C and the extracts dialysed in 10,000-12,000 MW cut-off dialysis tubing for 3-4 h against dialysis buffer (**Table 3**). Nuclear extracts were immediately aliquoted into 5-25 µL volumes and stored at -80°C.

Mammary Tissue

Murine mammary proteins were kindly provided by Dr. Yvonne Kuys (Dairy Science, AgResearch, Hamilton, NZ). Briefly, high salt extracts of nuclei were prepared essentially as described above. Frozen tissue was manually crushed in liquid nitrogen and thawed in 2 volumes of low salt buffer. The solution was homogenised with an *Ultra-Turrax T25* machine (Janke & Kunkel IKA®-Labortechnik) and filtered through cheese cloth. Cells collected in the filtrate were sheared in 1 mL low salt buffer containing 0.5% (v/v) NP-40 and nuclear proteins were extracted as described for COMMA-D cells. Alternatively, nuclear proteins were precipitated using ammonium sulphate as described by Schmitt-Ney *et al.*, (1991). Nuclei were isolated as described above and then proteins precipitating in a 10-65% ammonium sulphate cut were extracted.

In all cases, protein concentrations were determined by the method of Bradford (1976) using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, CA, USA) and bovine serum albumin as the standard.

(ii) Labelling of DNA -

Method 1 Double stranded oligonucleotides (AP-1 and Sp1, see **Table 5**) were purchased from Promega Corp., (WI, USA) and were labelled according to the manufacturer's instructions. Approximately 3.5 pmoles of DNA was incubated in polynucleotide kinase buffer, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and 8 U T4 polynucleotide kinase (Boehringer Mannheim NZ Ltd., Auckland) in a total volume of 10 μL for 10 min at 37°C. The reaction was stopped by addition of 1 μL 0.5 M EDTA and the volume was increased to 100 μL with TE. Approximately 35 fmoles (1 μL) of target DNA was used in each mobility shift assay reaction.

Method 2 Probes containing STAT5, YY1 and NF-κB binding sites (**Table 6**) were purchased as complementary single stranded oligonucleotides. Approximately 10-15 pmol of the complementary strands were annealed before use and 3' ends were filled in and labelled in the presence of 50 μM each of dATP, dGTP and dTTP, 1x Klenow buffer, 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) and 4 U Klenow (Boehringer Mannheim NZ Ltd., Auckland) in a total volume of 25 μL. Probes labelled in this way were gel purified by electrophoresis through 0.7 mm 6% polyacrylamide (40:1) gels to eliminate unincorporated radiolabelled nucleotides, single stranded or incompletely elongated DNA. Following electrophoresis, wet gels were covered in plastic and overlaid with *X-Omat AR* film to locate the radioactive double stranded oligonucleotides. DNA was eluted from the excised gel slice in 1 mL TE at 4°C overnight before precipitation with 0.1 volume 3 M sodium acetate, pH 5.3 and 2 volumes 96% (v/v) ethanol. DNA was centrifuged at 10,000 x *g* for 30 min and resuspended in 50 μL TE. Approximately 20,000 cpm (5-10 fmol) of target DNA was used in each mobility shift assay reaction.

Table 5 *Oligonucleotides*

Oligonucleotide	*Sequence*	*Source*
AP-1	5'..TTCCGGCTGACTCATCAAGCG AAGGCCGACTGAGTAGTTCGC	Promega Corp., WI, USA
m67 SIE	5'..GTCGACATTTCCCGTAAATC GTAAAGGGCATTTAGCAGCT	Gibco BRL, Akld, NZ
NF-κB	5'..AGTTGAGGGGACTTTCCCAGGC AAAGGGTCCG	Gibco BRL, Akld, NZ
Sp1	5'..ATTCGATCGGGGCGGGGCGAGC TAAGCTAGCCCCGCCCCGCTCG	Promega Corp., WI, USA
STAT5(30)	5'..TGTGGACTTCTTGGAATTAAGGGACTTTTG CCCTGAAAAC	Oligos Etc. Inc., OR, USA
STAT5(21)	5'..GGACTTCTTGGAATTAAGGGA TTAATTCCCT	Gibco BRL, Akld, NZ
STAT5(30-A)	5'..TGTGGACTTCTTGGAATT AGAACCTTAA	Gibco BRL, Akld, NZ
STAT5(30-B)	5'..ACTTCTTGGAATTAAGGGACTTTTG CCCTGAAAAC	Gibco BRL, Akld, NZ
YY1	5'..GGTTCTGATCGGCCATCTTGACTCC GGTAGAACTGAGG	Gibco BRL, Akld, NZ

(iii) Formation of DNA-protein complexes -

Nuclear protein extract (5 μg) was incubated with 10 mM Tris, pH 7.5/50 mM sodium chloride/1 mM magnesium chloride/0.5 mM EDTA/0.5 mM DTT/4% (v/v) glycerol/0.1 μg/μL poly (dI.dC)(dI.dC) (Boehringer Mannheim NZ, Ltd., Auckland) in a total volume of 19 μL and incubated for 10 min on ice. The labelled target DNA (20,000 cpm, 5-10 fmol) was then added in a volume of 1μL to each reaction, and the reaction was incubated for a further 10 min at RT. For competition assays, varying amounts (5-100 fold molar excess) of unlabelled competitor oligonucleotide were added to the reaction 20 min prior to addition of the probe. Alternatively, for antibody supershift reactions, 1 μg of antibodies were added to the reaction 45 min prior to addition of the probe. The antibodies used in supershifting experiments are listed in **Appendix 1**.

(iv) Gel analysis of DNA-protein complexes -

The DNA-protein complexes were subjected to electrophoresis in 6% non-denaturing polyacrylamide (40:1) gels in 0.5x TBE at 4°C. A constant current of 20 mA/gel was applied for approximately 1 h. Following electrophoresis, gels were dried at 70°C under vacuum and DNA-protein complexes were visualised by autoradiography. Gels were exposed to *X-Omat AR* film for 16-20 h at -70°C.

Ethics

All work using experimental animals was conducted with approval from the Ethics Committee on the Welfare of Experimental Animals (University of Waikato, Hamilton, NZ, Protocol No. 225) and the Ruakura Animal Ethics Committee (Ruakura Research Centre, Hamilton, NZ).

Chapter Three -
Identification of lactation-associated proteins

Complex regulatory factors control the lactation state of mammary epithelial cells. To date, several factors including STAT5, the C/EBPs, NF-1, single-stranded DNA binding factor, YY1 and the glucocorticoid receptor have been implicated in the regulation of the major milk protein genes at the level of transcription. However, in view of the complex and co-ordinated control of milk protein gene expression, additional factors are also likely to be involved, but have yet to be discovered. These could act at any of a number of levels including gene transcription, mRNA stability, translation, post-translational modification, protein degradation and secretion.

The identification of proteins which are altered in abundance in response to hormonal stimulation of mouse mammary epithelial cells can provide clues to the underlying molecular events regulating lactation. In particular, proteins rapidly synthesised in response to prolactin may mediate the pleiotropic effects of prolactin in mammary epithelial cells. In order to identify such proteins, the separation power of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) can be exploited in proteome analyses, to separate unknown proteins from complex protein mixtures for identification by amino acid sequencing. This approach was used in my Masters thesis to detect a number of hormonally-responsive proteins in COMMA-D cells and mouse mammary epithelial cells. However, these proteins were not identified (Beaton, 1995). Of particular interest, two proteins (p77 and p63) were increased in relative abundance with lactation state in the mouse and with prolactin treatment in COMMA-D cells. This chapter describes the identification of these two proteins and their assessment as potential regulators of lactation in mouse mammary epithelial cells.

Results

Identification of two lactation-associated and prolactin-responsive proteins, p77 and p66-

Proteome analyses designed to detect hormonally responsive proteins in COMMA-D cells and primary mouse mammary epithelial cells have revealed a number of proteins which are altered in relative abundance in response to prolactin and/or with lactation state (see **Fig. 8**, reproduced from Beaton, 1995). In particular, two proteins (p77 and p63, see **table 6**) were approximately two- to three-fold more abundant in mammary epithelial cells isolated from d 7 lactating mice compared with cells isolated from d 10 pregnant non-lactating mice. Furthermore, these proteins were also two- to three-fold more abundant in COMMA-D cells after three days of prolactin treatment compared with control cells cultured in the absence of prolactin (see **Fig. 9**).

Table 6 *Quantification of p77 and p63 protein levels in COMMA-D and mouse mammary epithelial cells*

Protein	pI	Subcellular location	Cell type	Integrated density*	Increase
p77	5.9	Cytosol	Primary cells	Preg 5.5 Lact 15.5, 15.9	2.8-fold
p77	5.9	Cytosol	COMMA-D cells	-Prl 5.5, 6.4 +Prl 15.5, 15.9	2.6-fold
p63	5.9	Cytosol	Primary cells	Preg 6.5 Lact 15.5, 18.1	2.6-fold
p63	5.9	Cytosol	COMMA-D cells	-Prl 6.5, 6.6 +Prl 15.5, 18.1	2.6-fold

* The integrated density of each protein spot was determined by densitometry (Bio-Rad *GS-690 Imaging Densitometer*, CA, USA) using *Molecular Analyst Densitometry* software (Bio-Rad Laboratories, CA, USA). Preg., cells isolated from pregnant mice; Lact., cells isolated from lactating mice; -Prl, control COMMA-D cells; +Prl, prolactin-treated COMMA-D cells; pI, approximate isoelectric point determined from two-dimensional polyacrylamide-SDS gels.

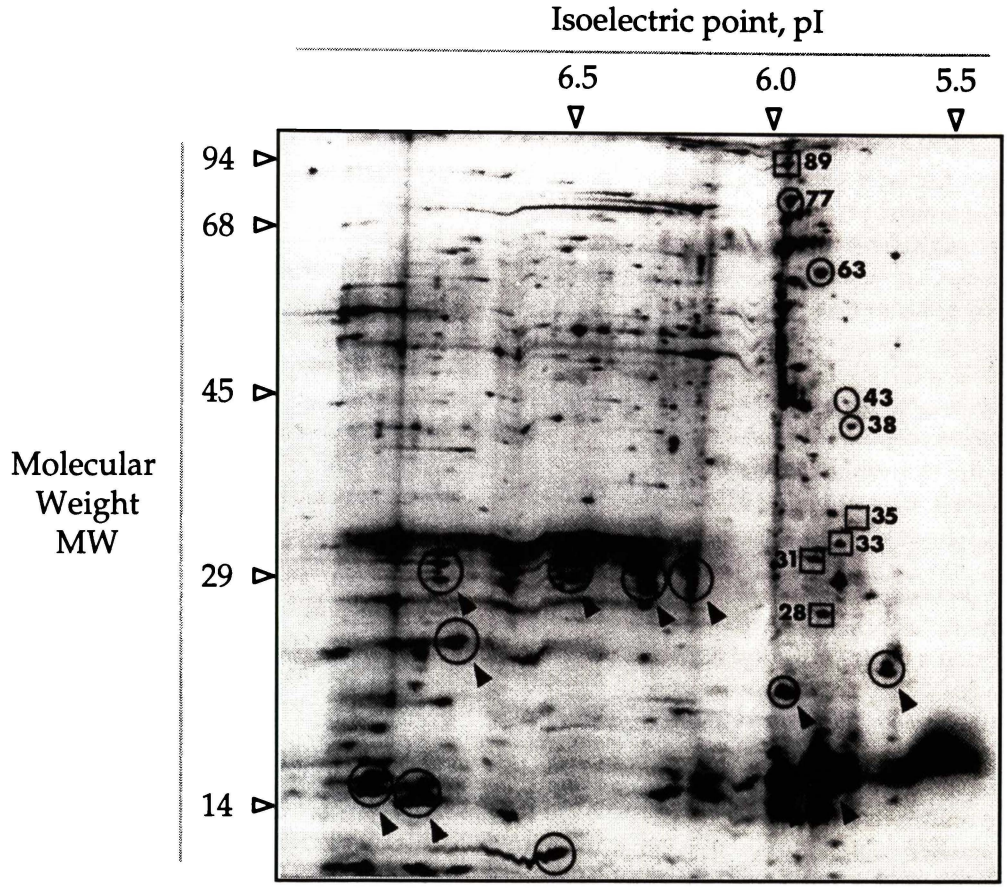


Figure 8 *Two-dimensional electrophoresis of cytosolic proteins from lactating mouse mammary epithelial cells.*

Mammary epithelial cells were isolated from lactating mice and metabolically labelled immediately after isolation in the presence of insulin and hydrocortisone. Cytosolic proteins derived from mouse mammary epithelial cells were separated by 2D-PAGE and visualised by autoradiography (20 d exposure). Circled proteins were increased and boxed proteins were decreased in relative abundance with lactation, compared with cells isolated from d 10 pregnant mice. Arrowed proteins reacted with anti-mouse milk antibodies. The numbers associated with protein spots refer to the estimated molecular weight of that protein in kilodaltons, kDa (reproduced from Beaton, 1995).

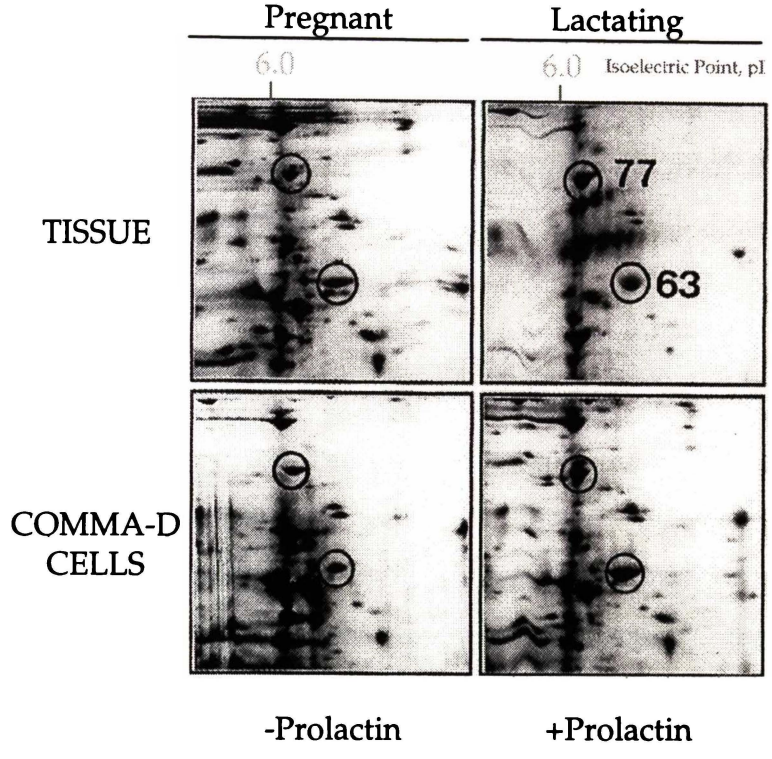


Figure 9 *Relative abundance of p77 and p63 in COMMA-D and mouse mammary epithelial cells.*

The panels show sections of two-dimensional polyacrylamide gels which were loaded with cytosolic proteins derived from COMMA-D or mouse mammary epithelial cells. COMMA-D cells were cultured and metabolically labelled in the presence of insulin, hydrocortisone and prolactin. Control cultures contained no prolactin. Alternatively, mammary epithelial cells were isolated from d10 pregnant or d 7 lactating mice, metabolically labelled and analysed as described in Fig. 8. Proteins were visualised by autoradiography (20 d exposure). p77 and p63 are circled.

Since p77 and p63 could be milk proteins, cytosolic proteins isolated from lactating mouse mammary epithelial cells were analysed by Western blotting, using antibodies raised against whole mouse milk. Eleven immunoreactive proteins were detected (arrowed in **Fig. 8**). These are likely to be isoforms of caseins (28-24 kDa), whey acidic protein (14 kDa) and α-lactalbumin (14 kDa). The two prominent and well resolved proteins, p77 and p63 were not among the immunoreactive proteins detected, indicating that they are not milk proteins.

By taking advantage of the high loading capacity of large format gels, both p77 and p63 were detected by Coomassie blue staining, thus allowing recovery from polyacrylamide gels in sufficient quantities for sequence analysis. The p77 and p63 protein spots were excised from 6 gels each loaded with 1 mg proteins (from the cytosolic fraction), and digested with *Endoproteinase Lys-C*. At least 35 proteolytic fragments of p77 and 30 of p63 were resolved by reverse-phase HPLC (**Fig. 10**). Two fragments of p77 and one of p63 (arrowed in **Fig. 10**) were subjected to amino acid sequencing. The two p77 fragments (NQLTSNPENT, TKPYIQVDIG) were homologous (10/10 and 10/10 amino acids) to murine glucose regulated protein 78 (grp78). These sequences are coloured in blue in **Fig. 11a**. Grp78 is found in the endoplasmic reticulum (ER) and is identical to the immunoglobulin heavy chain binding protein (Munro and Pelham, 1986). The p63 fragment (SNFEEALAAH) was homologous (10/10 amino acids) to murine protein disulphide isomerase (PDI). This sequence is coloured in blue in **Fig. 11b**. PDI is also found in the ER where it catalyses disulphide bond formation in a range of protein substrates (for a review see Freedman, 1989). Proteins that reside in the ER are characterised by the presence of a four amino acid carboxy terminus ER-retention motif (usually KDEL). This motif was located in the amino acid sequences of both grp78 and PDI (coloured in red in **Fig. 11**). The presence of ER proteins in the cytosolic extracts may be a result of ER disruption during subcellular fractionation in the presence of NP-40.

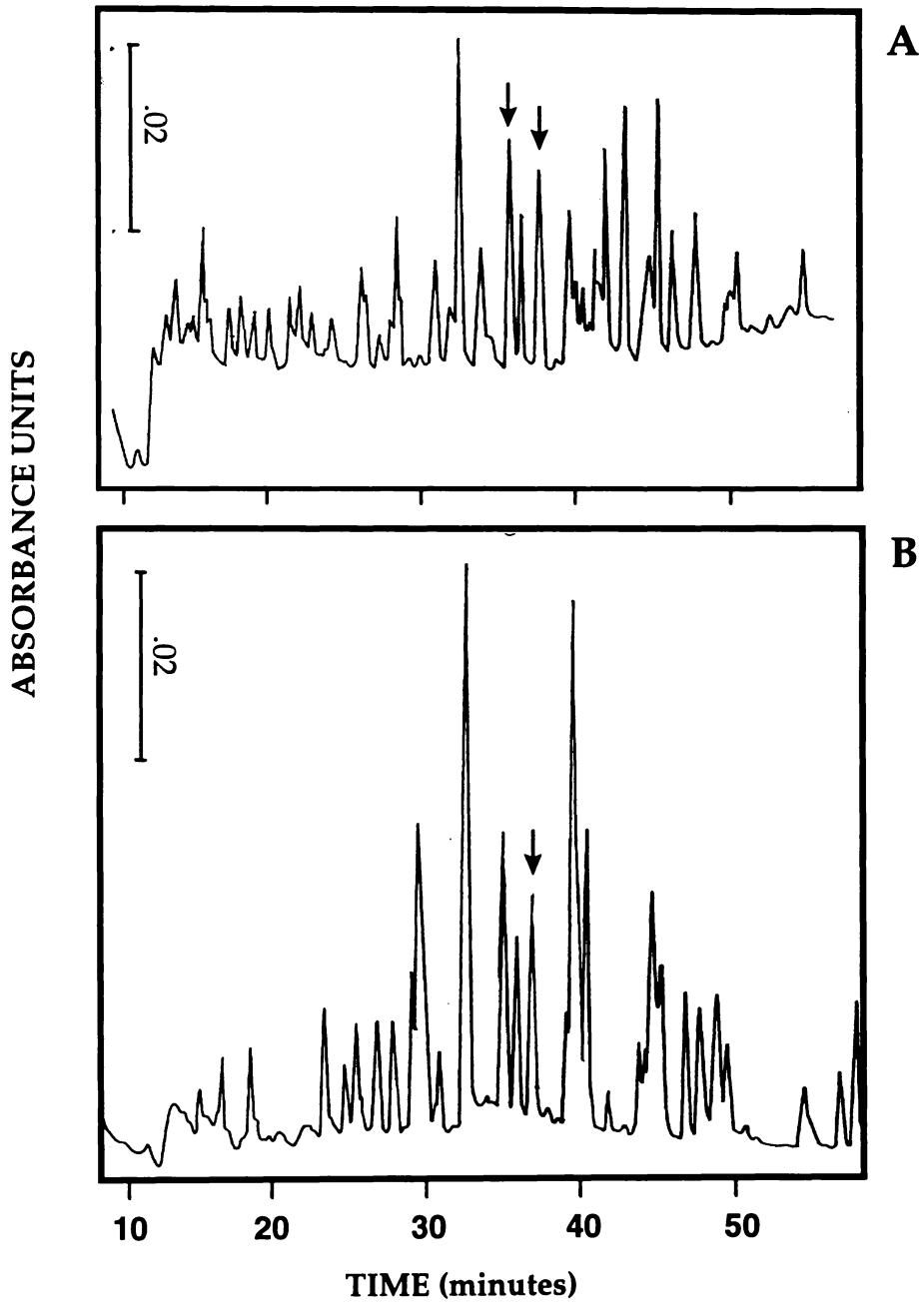


Figure 10 *Chromatograms of peptides obtained by enzymatic cleavage of proteins, p77 and p63*

Reverse-phase HPLC of *Lys-C* proteolytic digests of (A) p77 and (B) p63. Peptides were eluted with a 0-50% gradient of solvent B, where solvent B contains 80% (v/v) acetonitrile and 0.08% trifluoroacetic acid. Flow rate was 1 mL/min. Detector wavelength was 214 nm (0.1 Absorbance units full scale on the detector). Peptides selected for amino acid sequencing are indicated with arrows.

a) Glucose regulated protein 78 (grp78)

		↓	
MMKFTVVAAA	LLLLGAVRAE	EEDKKEDVGT	VVGIDLGTTY
SCVGVFKNGR	VEIIANDQGN	RITPSYVAFT	PEGERLIGDA
AK**NQLTSNPE**	**NT**VFDAKRLI	GRTWNDPSVQ	QDIKFLPFKV
VEKK**TKPYIQ**	**VDIG**GGQTKT	FAPEEISAMV	LTKMKETAEA
YLGKKVTHAV	VTVPAYFNDA	QRQATKDAGT	IAGLNVMRII
NEPTAAAIAY	GLDKREGEKN	ILVFDLGGGT	FDVSLLTIDN
GVFEVVATNG	DTHLGGEDFD	QRVMEHFIKL	YKKKTGKDVR
KDNRAVQKLR	REVEKAKRAL	SSQHQARIEI	ESFFEGEDFS
ETLTRAKFEE	LNMDLFRSTM	KPVQKVLEDS	DLKKSDIDEI
VLVGGSTRIP	KIQQLVKEFF	NGKEPSRGIN	PDEAVAYGAA
VQAGVLSGDQ	DTGDLVLLDV	CPLTLGIETV	GGVMTKLIPR
NTVVPTKKSQ	IFSTASDNQP	TVTIKVYEGE	RPLTKDNHLL
GTFDLTGIPP	APRGVPQIEV	TFEIDVNGIL	RVTAEDKGTG
NKNKITITND	QNRLTPEEIE	RMVNDAEKFA	EEDKKLKERI
DTRNELESYA	YSLKNQIGDK	EKLGGKLSSE	DKETMEKAVE
EKIEWLESHQ	DADIEDFKAK	KKELEEIVQP	IISKLYGSGG
PPPTGEEDTS	E**KDEL**		

b) Protein Disulphide Isomerase (PDI)

		↓	
MLSRALLCLA	LAWAARVGAD	ALEEEDNVLV	LKK**SNFEEAL**
AAHNYLLVEF	YAPWCGHCKA	LAPEYAKAAA	KLKAEGSEIR
LAKVDATEES	DLAQQYGVRG	YPTIKFFKNG	DTASPKEYTA
GREADDIVNW	LKKRTGPAAT	TLSDTAAAES	LVDSSEVTVI
GFFKDAGSDS	AKQFLLAAEA	VDDIPFGITS	NSDVFSKYQL
DKDGVVLFKK	FDEGRNNFEG	EITKEKLLDF	IKHNQLPLVI
EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	DGKLSNFKKA
AEGFKGKILF	IFIDSDHTDN	QRILEFFGLK	KEECPAVRLI
TLEEEMTKYK	PESDELTAEK	ITQFCHHFLE	GKIKPHLMSQ
ELPEDWDKQP	VKVLVGKNFE	EVAFDEKKNV	FVEFYAPWCG
HCKQLAPIWD	KLGETYKDHE	NIVIAKMDST	ANEVEAVKVH
SFPTLKFFPA	SADRTVIDYN	GERTLDGFKK	FLESGGQDGA
GDNDDLDLEE	ALEPDMEEDD	DQKAV**KDEL**	

Figure 11 *Amino acid sequence of murine glucose regulated protein 78 (grp78) and murine protein disulphide isomerase (PDI).*

The amino acid sequence deduced from nucleotide sequences of (a) grp78 and (b) PDI cDNA is shown. The amino acid sequences correspond to RNA extracted from mouse cells (Kozutsumi *et al.*, 1989; Gong *et al.*, 1988). The position of signal peptide cleavage is shown by an arrow for each sequence. Amino acids corresponding to sequenced peptides are coloured in blue and the endoplasmic reticulum-retention motif is coloured in red.

Grp78 and PDI gene expression in the mouse mammary gland-

To assess the biological relevance of mammary grp78, the relative abundance of grp78 was assessed in mammary tissue isolated from mice at various stages of the lactation cycle. Total mammary cellular proteins were isolated from a non-pregnant mouse, mice sacrificed during pregnancy (d 5 and d 16), at parturition, during lactation (d 1, d 3, d 4, d 5 and d 10) and involution (d 1, d 2, d 3 and d 4). Equivalent amounts of proteins were subjected to electrophoresis in a 10% (w/v) polyacrylamide-SDS gel and transfered to Polyscreen® PVDF membrane for Western analysis using anti-grp78 antibodies (see Materials and Methods, **table 4**). Grp78 protein levels remain relatively low during pregnancy and reach a maximal level at parturition (**Fig. 12**). This level was maintained throughout lactation. However, with the onset of involution, grp78 protein levels were dramatically reduced. This is consistent with the results of proteome analyses which showed a two-fold increase in mammary grp78 protein levels between day 10 of pregnancy and day 7 of lactation (**Fig. 9**). Antibodies were not available to investigate changes in the levels of PDI over the lactation cycle.

To determine whether the mRNA levels of grp78 and PDI reflect changes in the levels of these proteins, total RNA was isolated from mice at various stages of the lactation cycle. In the mouse, the mRNA levels of grp78 and PDI gradually increased during pregnancy, reaching a maximal level at late pregnancy/parturition (see **Fig. 13**), consistent with the observed increase in the abundance of these proteins. However, with the onset of lactation, the levels of grp78 and PDI transcripts decreased dramatically, and low levels were maintained throughout lactation and involution. To determine whether grp78 and PDI mRNA levels are hormonally responsive in the COMMA-D cell line, total RNA was also isolated from hormonally stimulated COMMA-D cells. In contrast to mammary tissue, the mRNA levels of grp78 and PDI gradually increased over time in response to prolactin (see **Fig. 14**). Grp78 and PDI mRNA levels were not rapidly induced in response to prolactin in COMMA-D cells or with lactation in the mouse. Therefore, these proteins are not likely to mediate rapid responses to prolactin in mammary cells.

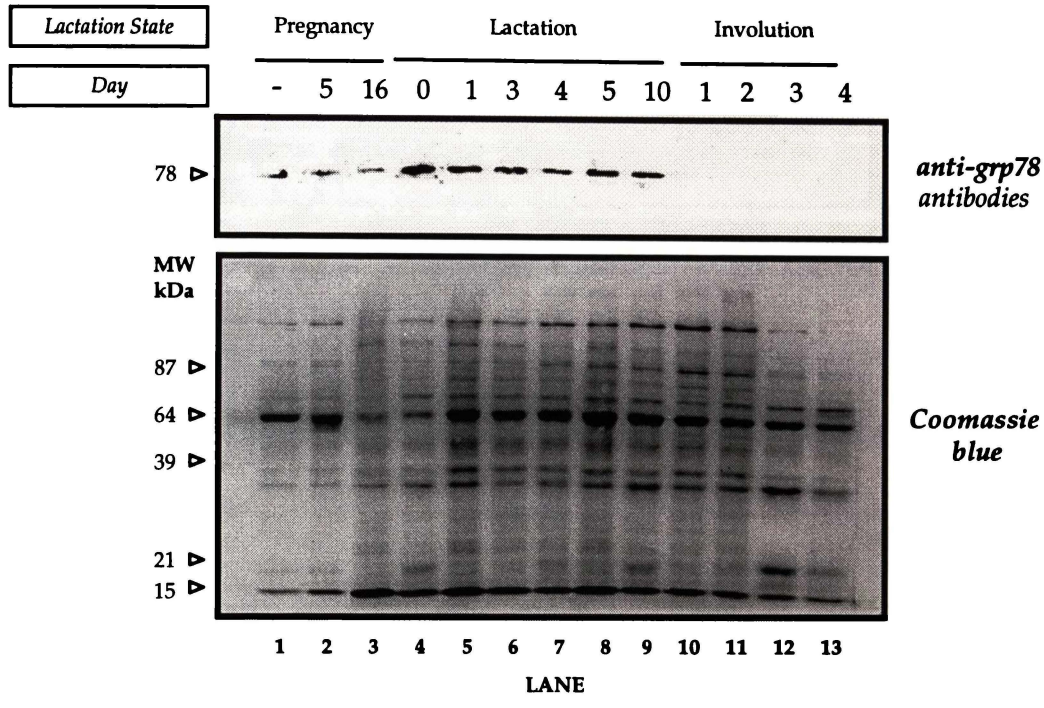


Figure 12 *Grp78 protein levels over the lactation cycle*

Equivalent amounts of total mammary cellular proteins (15 µg) isolated from a non-pregnant mouse, mice sacrificed during pregnancy (d 5 and d 16), at parturition, during lactation (d 1, d 3, d 4, d 5 and d 10) and involution (d 1, d 2, d 3 and d 4) were subjected to electrophoresis in a 10% polyacrylamide-SDS gel and transferred to Polyscreen® PVDF membrane for Western analysis. The blot was stained with Coomassie blue and then probed with anti-grp78 antibodies. The signal was detected using enhanced chemiluminesence (ECL) as described in Materials and Methods. MW, molecular weight in kilodaltons (kDa).

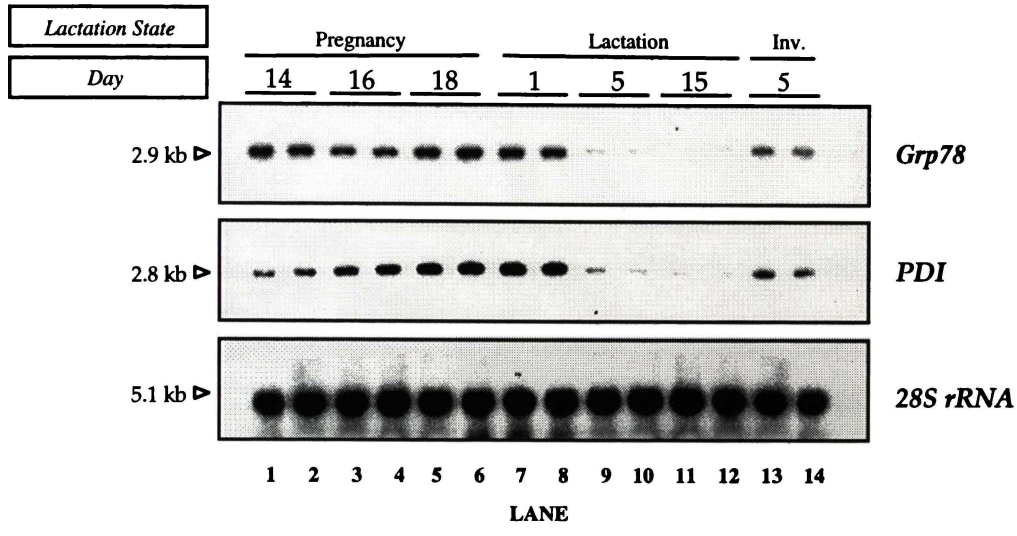


Figure 13 *Grp78 and PDI mRNA abundance over the lactation cycle.*

Equivalent amounts of mammary RNA (5μg) isolated from mice sacrificed during pregnancy (d 14, d 16 and d 18), lactation (d 1, d 5 and d 15) and involution (Inv., d 5) were subjected to electrophoresis in 1.2% (w/v) agarose/formaldehyde gels and blotted for Northern analysis. Probes were made by random primer labelling of grp78, PDI or ribosomal cDNA inserts from cloned plasmids, or PCR products (using [α-^{32}P]dCTP). Radiolabelled probes were hybridised to blots overnight at 65°C in phosphate/SDS hybridisation buffer as described in Materials and Methods. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

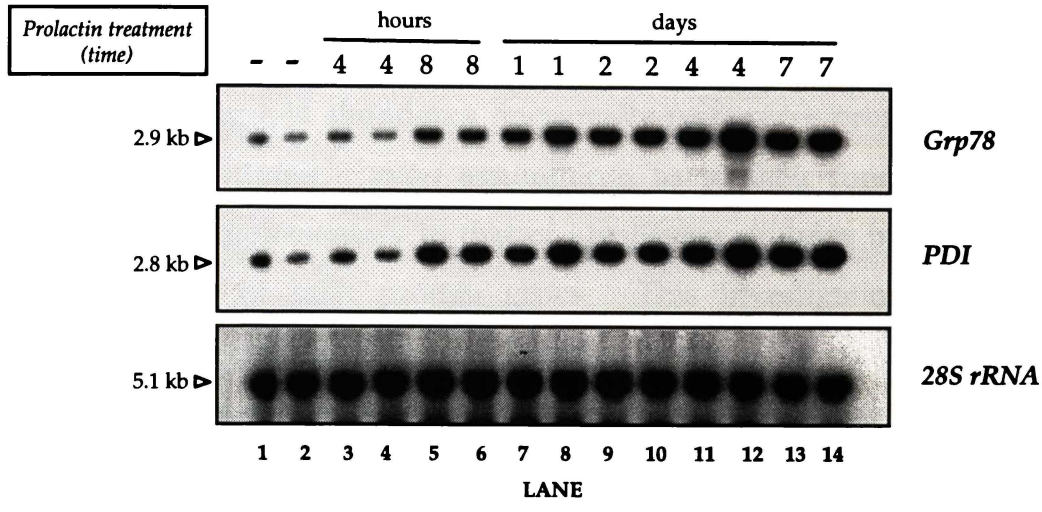


Figure 14 *Grp78 and PDI mRNA abundance in hormonally-stimulated COMMA-D cells*

COMMA-D cells were cultured on *Matrigel®*-coated plastic for various times (4 h - 7 d) in the presence of prolactin, insulin and hydrocortisone before isolation of total RNA. In addition, RNA was isolated from control COMMA-D cells cultured for 7 d in the absence of prolactin (lanes 1 & 2). Equivalent amounts of RNA (5µg) were subjected to electrophoresis in 1.2 % (w/v) agarose/formaldehyde gels and blotted for Northern analysis. Probes were made by random primer labelling of grp78, PDI or ribosomal cDNA inserts from cloned plasmids, or PCR products (using [α-^{32}P]dCTP). Radiolabelled probes were hybridised to blots overnight at 65°C in phosphate/SDS hybridisation buffer as described in Materials and Methods. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

Discussion

Two-dimensional electrophoresis has been successfully used in proteome analyses to detect proteins for identification in a range of biologically responsive systems (for examples see Mobbs *et al.*, 1990; Merrick *et al.*, 1994). When combined with subcellular fractionation this approach is a powerful way of detecting low abundance hormonally-responsive proteins. Proteins are analysed without *a priori* assumptions as to their function, thus the approach is complementary to that of identifying proteins interacting with the promoters of milk protein genes. This investigation focused on identifying two proteins that were found to be altered in relative abundance with lactation state in mouse mammary cells and in response to prolactin in the mouse mammary epithelial cell line, COMMA-D. Proteome analyses also revealed several other lactation-associated proteins in mouse mammary epithelial cells that were not prolactin-responsive in COMMA-D cells (Beaton, 1995, see **Fig. 8**). These proteins were not selected for sequence analysis because they were either present at very low abundance, or were not detected in both cell systems. However, since hormonally stimulated COMMA-D cells do not synthesise the full range of milk proteins to levels even approaching those in the mammary gland during lactation, these proteins may have a role regulating lactation in mammary epithelial cells *in vivo*. Further investigations would be required to investigate this possibility.

In the past, considerable emphasis has been placed on factors which regulate lactation at the level of transcription. However, although nuclear transcription factors are undoubtedly important, this does not preclude the involvement of proteins found elsewhere in the cell, which may act to regulate lactation at any of a number of different levels. The p77 (grp78) and p63 (PDI) proteins are of interest in this regard.

Grp78 and PDI are Reticuloplasmins

Grp78 and PDI are members of a family of multifunctional proteins called reticuloplasmins (for a review see Koch, 1987). Reticuloplasmins reside in the lumen of the endoplasmic reticulum (ER), and are characterised by a four residue ER-retention motif (usually KDEL) at the carboxy terminus of the protein. This four residue motif was found in the carboxy terminus amino acid sequence of both Grp78 and PDI. An amino terminal signal sequence facilitates entry of the preproteins to the ER where interaction between the KDEL motif and a specific receptor is thought to prevent transport of reticuloplasmins through the secretory pathway (Pelham, 1990; Scheel & Pelham, 1996). Reticuloplasmins appear to participate in a number of metabolic processes within the ER. For example, reticuloplasmins have been shown to exhibit molecular chaperone activity (Kuznetsov *et al.*, 1994), have an ability to bind calcium (Macer & Koch, 1988; Koch, 1990; Lucero *et al.*, 1994; Hubbard, 1996) and their abundance is increased in response to cellular stresses (Dorner *et al.*, 1990).

Glucose regulated protein 78 (Grp78)

To date, two major glucose regulated proteins (grp78 and grp94) have been identified (for a review see Little *et al.*, 1994). These proteins are found in many cell types and are particularly abundant in the ER of secretory cells. Glucose regulated proteins were originally detected in chick fibroblasts, and were found to be overproduced when fibroblasts were starved of glucose (Shiu *et al.*, 1977). The glucose regulated proteins have subsequently been found to be induced by a variety of substances; including tunicamycin, glucosamine, 2-deoxyglucose and calcium ionophores (Olden *et al.*, 1979; Resendez *et al.*, 1985; Welch *et al.*, 1983).

Although grp78 does not respond to heat shock (Pelham, 1986), it has approximately 60-70% amino acid identity with heat shock protein 70. The heat shock proteins are a small set of highly conserved proteins that are synthesised in response to heat shock and a number of other stresses in

prokaryotic and eukaryotic cells (for a review see Lindquist, 1986). It is thought that heat shock proteins protect cells from the effects of stress, possibly by disrupting aggregates that form after heat shock in the nucleus (Pelham, 1985), although the exact mechanisms are unclear. A 90 kDa heat shock protein, hsp90 is one of the major heat shock proteins and exists in cells under normal conditions (Lindquist & Craig, 1988). Hsp90 is thought to be involved in cellular growth and differentiation (Lindquist & Craig, 1988) via the regulation of casein kinase II (Miyata & Yahara, 1992) and steroid hormone receptors (Catelli *et al.*, 1985). Interestingly, the concentration of hsp90 was found to increase with lactation in bovine and murine mammary tissue (Watanabe *et al.*, 1997), and a role for hsp90 during mammary differentiation and lactation has been suggested. It is possible that glucose regulated proteins perform similar functions to heat shock proteins, but in response to other cellular stresses and within a different cellular compartment, the ER.

The relatively high abundance of grp78 in secretory cells and the structural similarity to the heat shock proteins has led to speculation that it might act as a molecular chaperone for secreted proteins in the ER lumen (Munro & Pelham, 1986; Nigam *et al.*, 1994). This is strongly supported by the finding that grp78 and immunoglobulin heavy chain binding protein (BiP) are encoded by the same gene (Kozutsumi *et al.*, 1989). BiP associates with the exposed hydrophobic regions of newly synthesised immunoglobulin heavy chains in the ER to prevent their aggregation, thereby facilitating the assembly of immunoglobulins (Bole *et al.*, 1986). Grp78 has also been reported to tightly associate with other newly synthesised proteins in the ER that are incompletely assembled, have mutant structures or are incorrectly glycosylated (Kassenbrock *et al.*, 1988). Since grp78 itself is retained in the ER lumen and not transported through the secretory pathway, proteins bound to it will also be retained. This would provide a potential mechanism for preventing the transport of malfolded or mutant proteins to the cell surface.

Protein Disulphide Isomerase (PDI)

Like grp78, protein disulphide isomerase (PDI) has been reported to exhibit molecular chaperone activity (Puig *et al.*, 1994a; 1994b). However, unlike grp78, PDI also catalyses the rearrangement of disulphide bonds in various proteins *in vitro* and is considered to be the *in vivo* catalyst for disulphide bond formation in secreted proteins (for a review see Freedman, 1989). Interestingly, rat PDI cDNA sequences were found to be highly homologous to those determined for the β-subunit of human prolyl 4-hydroxylase, a key enzyme in collagen biosynthesis. Southern analysis subsequently showed that the β-subunit and PDI are products of the same gene (Pihlajaniemi *et al.*, 1987). The active enzyme consists of an $\alpha_2\beta_2$ tetramer and catalyses the formation of 4-hydroxyproline in collagen by the hydroxylation of proline residues in peptide linkages. The reaction product, 4-hydroxyproline stabilises collagen triple helices under physiological conditions (for a review see Pihlajaniemi *et al.*, 1991). It is not clear whether the β-subunit/PDI is catalytically involved in the hydroxylation reaction (Kivirikko *et al.*, 1989). However, since the β-subunit/PDI possesses the four amino acid carboxy terminus ER-retention motif but the α-subunit lacks this signal, one function of the β-subunit may be to retain this enzyme in the ER. PDI is also identical to a major cellular thyroid hormone-binding protein (Cheng *et al.*, 1987; Yamauchi *et al.*, 1987) and is a component of the microsomal triglyceride transfer protein complex (Wetterau *et al.*, 1990). Current findings suggest that PDI functions to maintain the transfer protein in a nonaggregated, catalytically active form (Wetterau *et al.*, 1991). Therefore, in addition to catalysing proper folding of newly synthesised disulphide-bonded proteins, PDI may play a wider role as part of heteroligomeric enzymes such as prolyl 4-hydroxylase and microsomal triglyceride transfer protein in maintaining their active structure.

Regulation of grp78 and PDI gene expression

In this study, the synthesis of grp78 and PDI proteins were shown to be co-ordinately induced in mouse mammary epithelial cells following hormonal stimulation. This has also been observed by others (Hensel *et al.*, 1994). The synthesis of both grp78 and PDI were increased to the same degree in the rat pancreas following hormonal stimulation of secretion. Therefore, grp78 and PDI appear to have co-ordinated functions in at least one exocrine tissue in addition to the mammary gland. The co-ordinate regulation of glucose regulated genes in mammary cells has been reviewed by Lee, (1987).

A co-ordinated response to hormonal stimulation was also observed in the mRNA levels of grp78 and PDI in COMMA-D cells and the mouse mammary gland over the lactation cycle. This would seem to indicate that the co-ordinated hormonal response may be integrated at the transcriptional level. While previous studies have established that a highly conserved sequence in the grp78 promoter, termed the *grp* core element is one of the key regulatory elements required for the induction of gene expression (Resendez *et al.*, 1988; Li *et al.*, 1994), the identities of the factors which bind to this region are unknown. However, recent evidence suggests that the nuclear factor YY1 is capable of binding to the conserved stress-inducible *grp* core element under conditions of Ca^{2+} depletion and formation of aberrant proteins to activate transcription (Li *et al.*, 1994; 1997). In addition to the core element, the promoter regions of the *grp78* and *PDI* genes contain a series of CCAAT-like elements, GC-rich motifs and other potential regulatory elements (Wooden *et al.*, 1991; Lin *et al.*, 1986; Tasanen *et al.*, 1992). This level of complexity may allow interactions between a number of transcription factors, resulting in the induction of these genes in response to a wide range of stimuli, possibly including lactogenic hormones.

In this study, the highest levels of grp78 and PDI transcripts were detected during late pregnancy in mouse mammary tissue, but the highest levels of

these proteins were observed during lactation. These patterns of expression are similar to those seen with the 90 kDa heat shock protein, Hsp90 (Catelli *et al.*, 1989). This may indicate the importance of post-transcriptional regulation for the expression of these genes. Catelli *et al.*, (1989) speculate that stability of the hsp90 protein was increased in the lactating mammary gland because of the decrease of some of the endogenous proteases (Ossowski *et al.*, 1979). This may also be the case for grp78 and PDI. Alternatively, the decrease in the abundance of transcripts for these genes could be the result of a dilution effect due to the high levels of milk protein gene transcripts in the lactating gland and/or the increased synthesis of ribosomal RNA. This is consistent with the slight increase in the levels of grp78 and PDI transcripts detected during involution, in the absence of a dilution effect and increased synthesis of ribosomal RNA. Further investigations beyond the scope of this thesis would be required to verify these hypotheses, and elucidate the exact mechanisms involved in regulating these genes at both the transcriptional and post-transcriptional levels.

Grp78 and PDI in the mammary gland

In this study, grp78 and PDI were shown to be prolactin-responsive in COMMA-D cells and lactation-associated in the mouse mammary gland. Increased levels of these proteins have also been detected by others in lactating mammary tissue. These observations suggest that these proteins may have a functional role during lactation. Ghosal *et al.*, (1994) investigated the intracellular distribution of these and other reticuloplasmins in the rat mammary gland during the lactation cycle. In particular, PDI was abundant in intracellular lipid droplet precursors of milk fat globules in lactating tissue. However, only barely detectable quantities of PDI were detected in milk lipid globule membrane, suggesting that it is lost from intracellular lipid droplets before or during their secretion as milk lipid globules. Immunocytochemical localisation confirmed the presence of PDI on intracellular lipid droplets and in non-ER regions of cells. Thus, in addition to processing proteins for secretion, it is thought that PDI may have a role in

lipid droplet assembly during lactation. Since mammary cells synthesise copious proteins and fats for secretion, which undergo significant co- and post-translational modifications within the ER lumen, co-ordinate molecular chaperone functions for grp78 and PDI could be envisaged in order to facilitate these continual processes.

Finally, the ability of grp78 and PDI to bind calcium is particularly relevant to mammary cell physiology (Macer & Koch, 1988). Milk calcium exists in bound forms, associated with casein micelles and complexed to citrate and phosphate, as well as ionised forms. Interestingly, ionised calcium in milk is thought to be in the concentration range of 1-4 mM, at least 1000 times its postulated concentration in the mammary cell (Neville & Watters, 1983). It is possible that grp78 and PDI are part of a group of proteins that facilitate the transport and storage of large amounts of calcium by binding and regulating the mobilisation of calcium in the ER, to regulate calcium-dependent protein folding and transport (Lodish *et al.*, 1992).

Future directions

While many approaches are directed towards detecting changes in gene expression during lactogenic signalling, *de novo* protein synthesis may not be required to elicit the immediate effects of prolactin in mammary epithelial cells. For example, these signals may be solely mediated by pre-existing proteins which are activated via post-translational modifications, such as for STAT5, a mediator of the prolactin activated JAK-STAT pathway. The ability to detect post-translational modifications to proteins is one advantage of analysing proteins using 2D-PAGE. Since this technique separates proteins according to isoelectric point in the first dimension and molecular weight in the second dimension, any post-translational modifications which alter the net charge of a protein (such as changes to the phosphorylation state) can be detected. Nevertheless, not all proteins are detected using large format 2D-PAGE in proteome analyses. This may be because they are of very low abundance, very basic or acidic, synthesised at very low rates, or they are

expressed only transiently in response to prolactin. Thus, proteome analyses alone are not comprehensive, and other complementary approaches should also be used. For example, lactation-associated changes in mammary gene expression could be detected using techniques such as representational difference analysis, differential display and subtractive hybridisation. Essentially, each of these methods attempts to detect differences in mRNA species isolated from different populations of cells, for example cells isolated from lactating and non-lactating animals. In addition, promoter analyses which characterise and identify proteins interacting with the promoters of the milk protein genes could be conducted. However, identifying a requirement for *de novo* protein synthesis during the hormonal induction of milk protein gene expression would provide a rational basis to use such techniques, in order to identify potential regulators of lactation in mouse mammary epithelial cells.

Chapter Four -
Hormonal induction of β-casein gene expression: Is there a requirement for ongoing protein synthesis?

At the cellular level, milk protein gene expression is regulated by a myriad of hormonal and other extracellular signals, including the lactogenic hormone prolactin. Prolactin's signals are mediated via the JAK2-STAT5 pathway, and the mitogen-activated protein kinase (MAPK) pathway (which involves signalling through the SHC/Sos/Grb2/*ras*/*raf*/MAPK cascade). Although lactogenic hormone treatment stimulates the rapid activation of MAPK in mammary cells, the MAPK pathway does not appear to contribute to hormonal activation of β-casein gene expression (Wartmann *et al.*, 1996). Thus, the JAK2-STAT5 pathway appears to be the major signalling route from the cell membrane to the nucleus for activation of milk protein gene expression.

Composite response elements have been identified in the promoter regions of a number of milk protein genes and contain multiple binding sites for several different transcription factors (Rosen *et al.*, 1998). Cooperation between different transcription factors at sites within these elements may provide a mechanism for distinct signalling pathways to converge at the promoters of the milk protein genes. Therefore, due to the nature of composite response elements, a number of factors are involved in the transcription of the milk protein genes, some of which may not have been identified. Although *de novo* synthesis of STAT5 is not required to transmit prolactin's signal (Gouilleux *et al.*, 1994), the synthesis of other proteins may be required to mediate the pleiotropic effects of prolactin and other signals in mammary cells. This chapter describes experiments designed to determine whether ongoing protein synthesis is necessary for prolactin-stimulated β-casein gene expression in mammary cells.

Results

(i) Effect of protein synthesis inhibition on β-casein gene expression in mouse mammary epithelial cells-

Cycloheximide (CHX) blocks protein synthesis by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit (Hogan, 1969; Fan & Penman, 1970). To determine the optimum concentration of CHX required for effective inhibition of protein synthesis in cultured cells, COMMA-D cells were cultured in the presence of prolactin and CHX for 8 h and metabolically labelled with ^{35}S[methionine] for the final 3 h of this incubation period (as described in Materials and Methods). The trichloroacetic acid (TCA) precipitable counts incorporated into proteins were measured in cells cultured in the presence of five different concentrations of CHX. The values for counts incorporated into cellular proteins are expressed as an average of three replicates (± half the range), and protein synthesis in the presence of CHX is expressed as a percentage of protein synthesis in control cells (**Table 7**). The concentration of CHX which inhibited protein synthesis in COMMA-D cells by 95% (25 μM or 40 μg CHX/ml culture medium) was used for all further experiments in this study. This concentration of CHX is similar to that used by others (Greenburg *et al.*, 1986; Goodman & Rosen, 1990; Yoshimura & Oka, 1990).

The effect of protein synthesis inhibition on β-casein mRNA accumulation was initially tested in the COMMA-D cell line. Cells were cultured in the presence of insulin and hydrocortisone for 2 d, and then for 8 h in the presence or absence of prolactin and CHX (see **Fig. 15a**). CHX was added to the cell culture medium from 15 min prior to the addition of prolactin, in order to fully inhibit protein synthesis during lactogenic signalling. Following prolactin treatment, cells were harvested and total RNA was isolated for Northern analysis (as described in Materials and Methods).

Table 7 *The effect of CHX on cellular protein synthesis*

CHX concentration (μM)	*Counts incorporated**	*Protein synthesis*$^{\Psi}$
0	109,372 ± 29,075	100 %
6.25	8,471 ± 706	7.7 %
12.5	7712 ± 1518	7.1 %
25	5,496 ± 746	5.0 %
50	4,103 ± 283	3.8 %
100	3,230 ± 642	3.0 %

* Values are an average of three replicates (± half the range)
$^{\Psi}$ Expressed as a percentage of protein synthesis in control cells

The addition of prolactin to the cell culture medium stimulated the accumulation of β-casein transcripts (**Fig. 15b**, compare lanes 1 & 3). However, when COMMA-D cells were cultured in the presence of both prolactin and CHX, the accumulation of β-casein mRNA was substantially reduced, compared with prolactin alone (lanes 3 & 4). The levels of β-casein transcripts were quantified by densitometry (normalised to 28S rRNA). The increase in β-casein transcripts was inhibited by approximately 95% during the 8 h treatment with CHX.

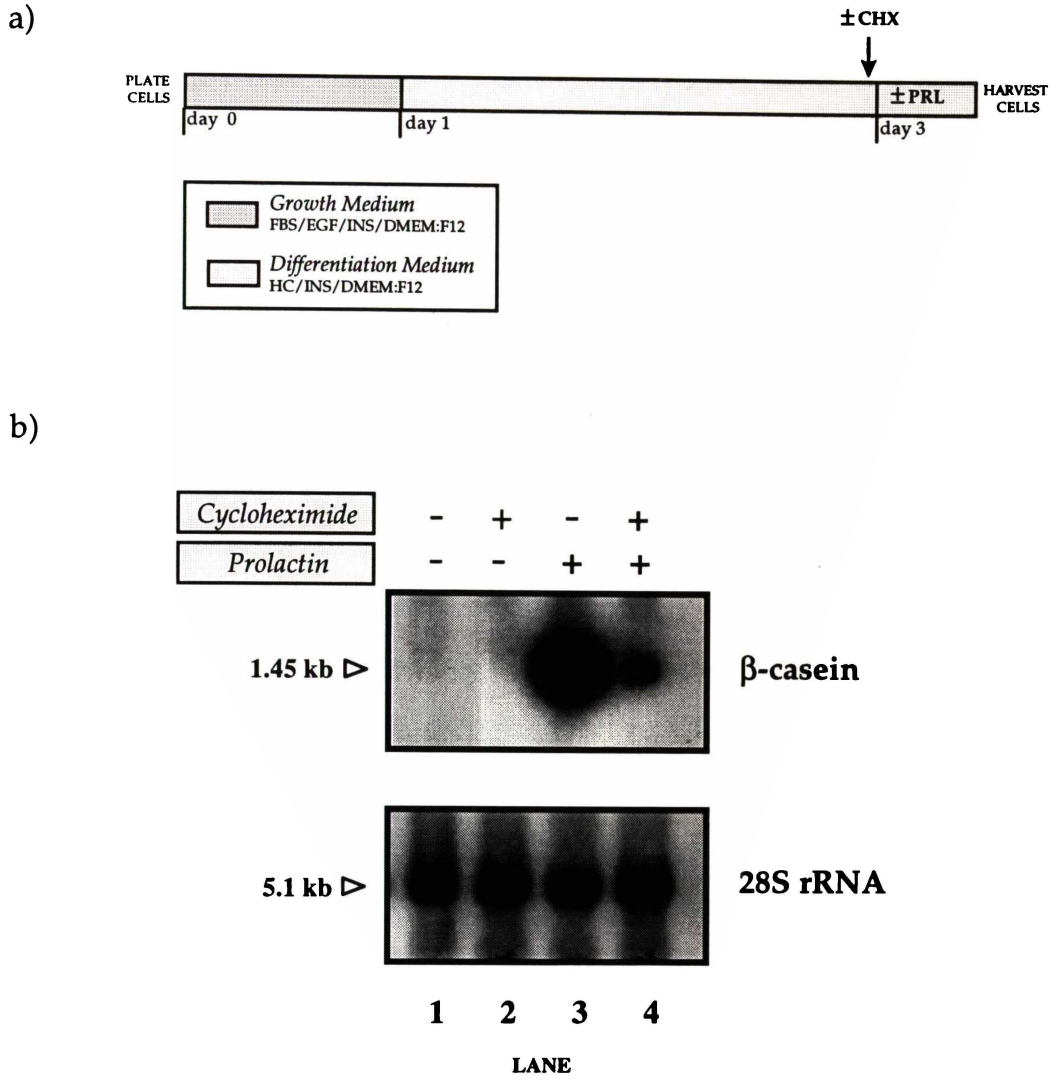


Figure 15 *Effect of CHX on the induction of β-casein mRNA accumulation in COMMA-D cells*

a) COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone, and then for 8 h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. CHX was added to the culture medium from 15 min prior to the addition of prolactin.

b) Following hormonal stimulation, COMMA-D cells were harvested, total RNA was isolated and equivalent amounts of RNA (5 μg) were subjected to electrophoresis in a 1.2% (w/v) agarose/1.8% (v/v) formaldehyde gel and blotted for Northern analysis. Probes were made by random primer labelling (with [^{32}P]dCTP) of β-casein or ribosomal cDNA inserts from cloned plasmids. Radiolabelled probes were hybridised to blots overnight at 65°C in phosphate/SDS hybridisation buffer. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left. CHX, cycloheximide; DMEM:F12, Dulbecco's Modified Eagle's medium:F12 nutrient mix; EGF, epidermal growth factor; FBS, foetal bovine serum; HC, hydrocortisone; INS, insulin; PRL, prolactin.

To confirm that the CHX-dependent decrease in β-casein mRNA levels was due to a general effect on protein synthesis, the effect of a functionally distinct protein synthesis inhibitor was also studied. Anisomycin inhibits the association of ribosomal subunits (Lewis & Mathews, 1980; Greenburg *et al.*, 1986). COMMA-D cells were cultured for 2 d in the presence of insulin and hydrocortisone, and then for 8 h in the presence or absence of prolactin and/or anisomycin. Following prolactin treatment, total RNA was isolated for Northern analysis. When prolactin and anisomycin were both added to the culture medium, the accumulation of β-casein mRNA was substantially reduced, compared with prolactin alone (**Fig. 16,** compare lanes 3 & 4). Thus, both protein synthesis inhibitors elicited similar effects on β-casein mRNA accumulation, strongly suggesting that ongoing protein synthesis is required for maximal β-casein mRNA accumulation in COMMA-D cells during the first 8 hours of prolactin treatment.

The effect of CHX on β-casein mRNA accumulation was also examined in primary mouse mammary epithelial cells, to test the requirement for ongoing protein synthesis in mammary cells other than the COMMA-D cell line. Mammary epithelial cells were isolated from d 14-15 pregnant mice and cultured in the presence of insulin and hydrocortisone for 2 d. Cells were then cultured with or without prolactin and/or cycloheximide for 8 h. Following prolactin treatment, cells were harvested and total RNA was isolated for Northern analysis. The addition of prolactin to the culture medium resulted in a significant increase in β-casein mRNA levels, compared with the background levels of β-casein transcripts detected in cells isolated from the pregnant gland (**Fig. 17,** compare lanes 1 & 3). When primary cells were cultured in the presence of both prolactin and cycloheximide, the accumulation of β-casein mRNA was substantially reduced, compared with prolactin alone (lanes 3 & 4). Furthermore, a CHX-dependent decrease in the background levels of β-casein transcripts was detected in cells cultured in the absence of prolactin (lanes 1 & 2).

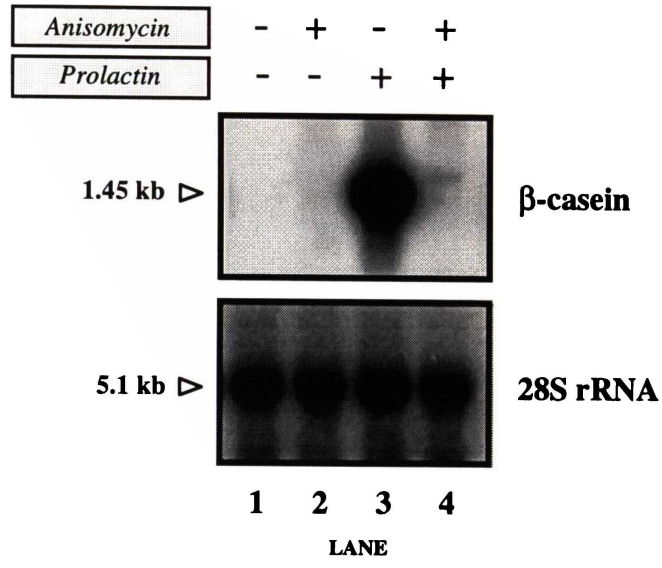


Figure 16 *Effect of anisomycin on the induction of β-casein mRNA accumulation in COMMA-D cells*

COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone, and then for 8 h in the presence or absence of 5 μg/mL prolactin and/or 2.7 μg/mL (10 μM) anisomycin. Anisomycin was added to the culture medium from 15 min prior to the addition of prolactin. Cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

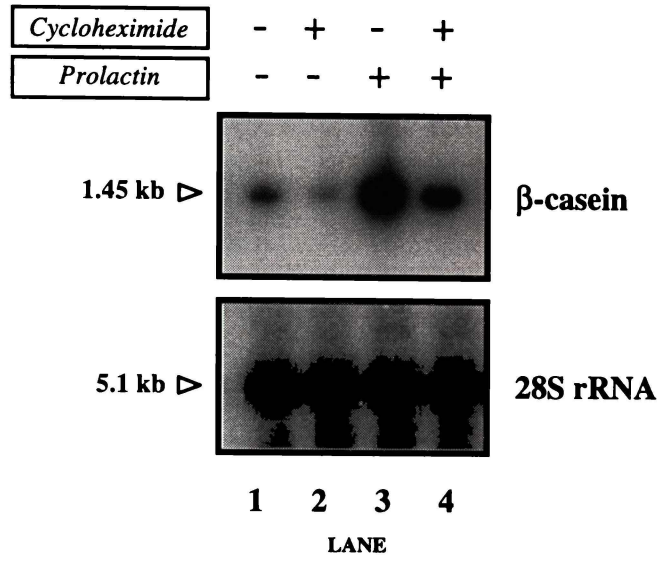


Figure 17 *Effect of CHX on the induction of β-casein mRNA accumulation in primary mouse mammary epithelial cells*

Primary mouse mammary epithelial cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone, and then for 8 h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. CHX was added to the culture medium from 15 min prior to the addition of prolactin. Cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

These results show that protein synthesis is also required in primary cells for maximal accumulation of β-casein transcripts. Since COMMA-D cells do not synthesise all of the milk proteins in response to prolactin, the effect of CHX on the accumulation of other milk protein transcripts was examined in primary cells. The same mRNA samples were probed for expression of the α-lactalbumin and whey acidic protein genes. These messages were not detected in hormonally stimulated cells (results not shown).

To determine whether protein synthesis is required to mediate rapid responses to prolactin, COMMA-D cells were cultured for 2 d in the presence of insulin and hydrocortisone, and then in the presence of prolactin for up to 2 h. β-casein trancripts were first detected in COMMA-D cells between one and two hours after the addition of prolactin to the culture medium (**Fig. 18**). The effect of CHX on β-casein mRNA accumulation was then examined at these time points to determine whether protein synthesis is required for the rapid activation of gene expression. In the presence of CHX, β-casein mRNA accumulation is reduced from the earliest time that β-casein mRNA can be detected, compared with prolactin alone (**Fig. 19**, lanes 4 & 5). β-casein transcripts accumulated to low levels after 2 h in the presence of prolactin and CHX but did not increase any further over the following 6 h (lanes 5, 7 & 9). When the addition of CHX to the culture medium was delayed by 4 h, any further increase in the level of β-casein mRNA was prevented in prolactin-treated cells (**Fig. 20**, lanes 3-6), suggesting that protein synthesis is also required for the maintenance of β-casein gene expression at a maximal level. Thus, protein synthesis is required for the activation and maintenance of β-casein gene expression.

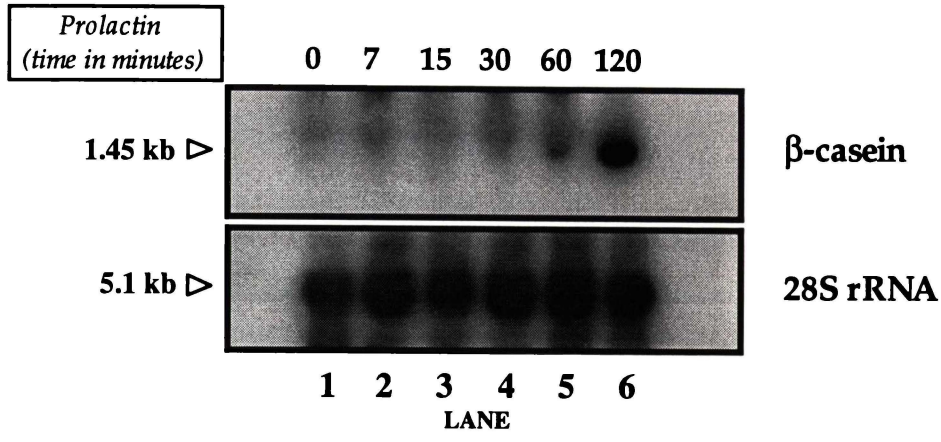


Figure 18 *Prolactin induced accumulation of β-casein mRNA in COMMA-D cells* COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone and then in the presence or absence of 5 μg/mL prolactin. Cells were harvested at various time points following the addition of prolactin, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis, and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left

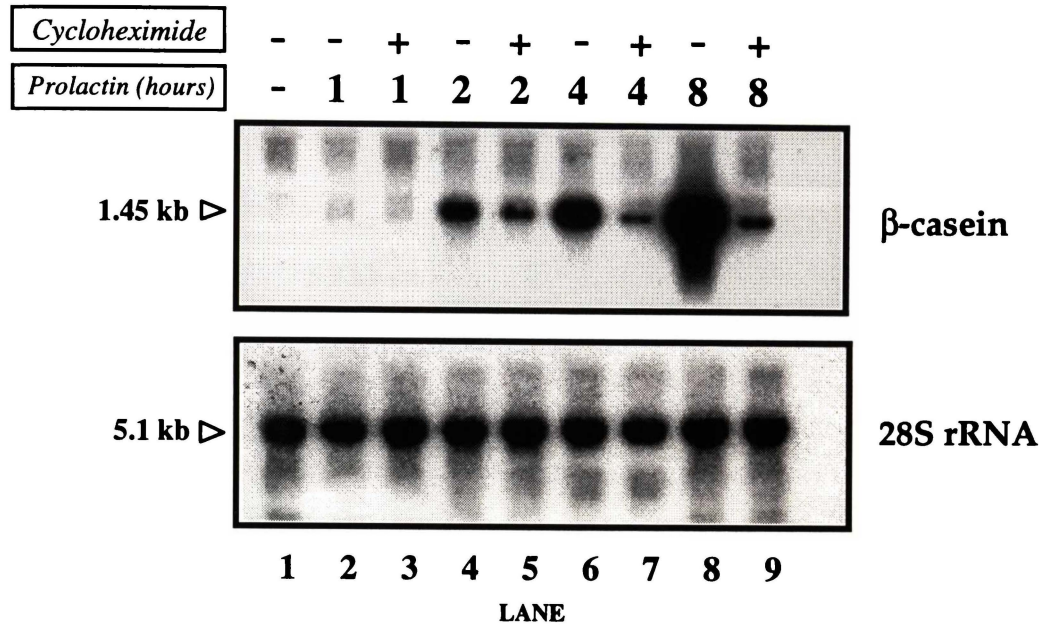
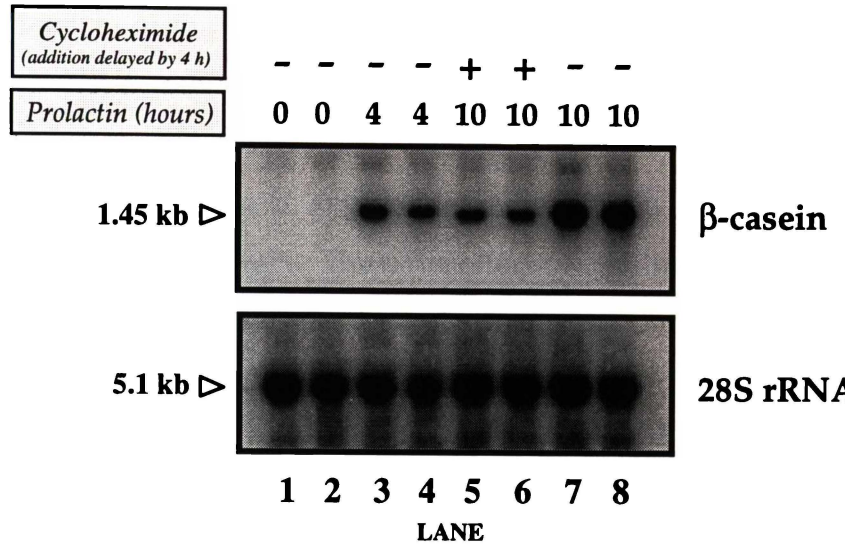


Figure 19 *The effect of CHX on the induction of β-casein mRNA accumulation in COMMA-D cells over time*

COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone, and then in the presence of 5 μg/mL prolactin and/or 40 μg/mL CHX. Cells were harvested at various time points following the addition of prolactin, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

a)



b)

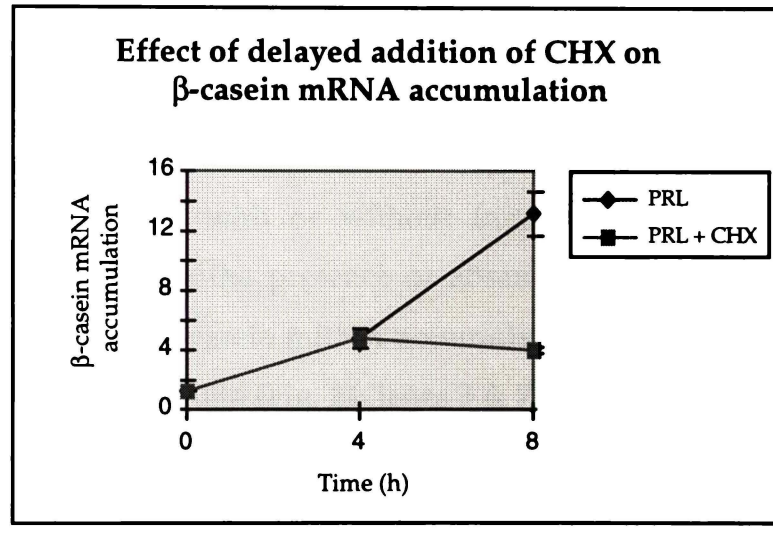


Figure 20 *The effect of delayed addition of CHX on the induction of β-casein mRNA accumulation in COMMA-D cells*

a) COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone, and then for 10 h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. The addition of CHX to the culture medium was delayed by 4 h. Following the 10 h incubation period, cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

b) Results are presented as the amount of β-casein mRNA detected in cells cultured in the absence or presence of CHX (addition delayed by 4 h), and are the means ± half the range of duplicate estimations. Where no *error bars* are apparent they fall within the *symbol*.

(ii) Is the effect of CHX on β-casein gene expression dependent on other extracellular stimuli?

Since prolactin regulates milk protein gene expression in conjunction with other hormones and extracellular stimuli, experiments conducted to examine the role of prolactin in regulating milk protein gene expression should also consider these factors. Thus, the effect of insulin, hydrocortisone and the extracellular matrix on CHX-dependent suppression of β-casein gene expression were examined in COMMA-D cells.

Insulin

Insulin is known to elicit a wide range of biological responses, such as stimulating growth and differentiation of cells, and in particular it has also been implicated in the activation of milk protein gene expression (Houdebine *et al.*, 1985). To determine whether the effect of CHX is dependent on the lactogenic hormone, insulin, COMMA-D cells were cultured for 2 d in the presence of hydrocortisone, with or without insulin. Cells were then cultured for a further 8 h in the presence or absence of prolactin and/or CHX. Prolactin stimulates β-casein mRNA accumulation to similar levels in the presence or absence of insulin (**Fig. 21**, lanes 3 & 7). The CHX-dependent decrease in β-casein transcripts was observed in the presence or absence of insulin (lanes 3-4 & 7-8). These results indicate that the effect of protein synthesis inhibition on β-casein gene expression is independent of insulin.

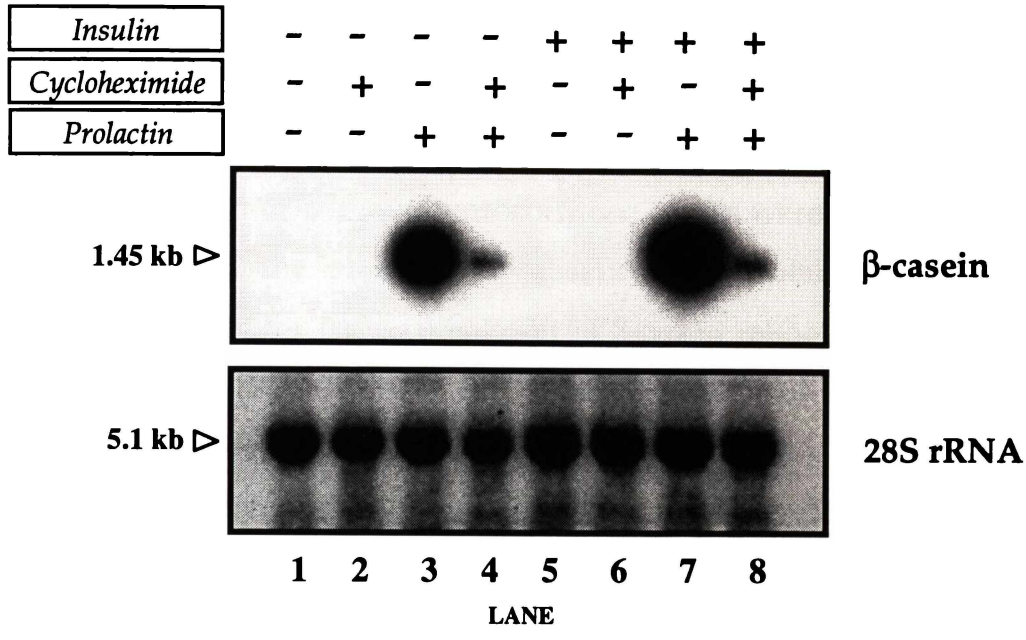


Figure 21 *Effect of insulin on CHX-induced suppression of β-casein mRNA accumulation in COMMA-D cells*

COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of hydrocortisone and with or without insulin. Cells were then cultured for 8 h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. CHX was added to the culture medium from 15 min prior to the addition of prolactin. Cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

Glucocorticoids

Glucocorticoids are known to synergise with prolactin for maximal expression of milk protein genes. To determine whether the effect of CHX on β-casein mRNA accumulation is dependent on the lactogenic hormone, hydrocortisone, COMMA-D cells were cultured for 2 d in the presence of insulin and with or without hydrocortisone. Cells were then cultured for a further 8 h in the presence or absence of prolactin and/or CHX. Prolactin stimulates the accumulation of β-casein mRNA in the presence of hydrocortisone (**Fig. 22**, lane 7). However, the level of β-casein mRNA accumulation was substantially reduced in prolactin-treated cells cultured in the absence of hydrocortisone (compare lanes 3 & 7). This result is consistent with previous studies (Guyette *et al.*, 1979; Doppler *et al.*, 1989). A CHX-dependent decrease in β-casein transcripts was observed in prolactin-treated COMMA-D cells cultured in the presence or absence of hydrocortisone (**Fig. 22**, lanes 3-4 & 7-8). No transcripts were detected in prolactin- and CHX-treated cells cultured in the absence of hydrocortisone (lane 4). These results indicate that the effect of protein synthesis inhibition on β-casein gene expression is also independent of the lactogenic hormone, hydrocortisone.

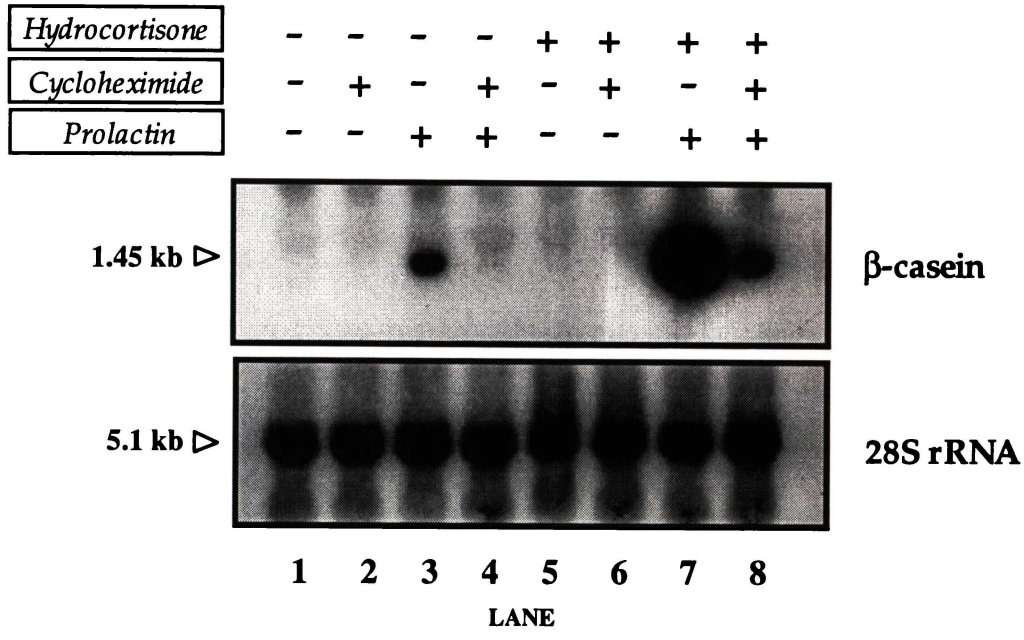


Figure 22 *Effect of hydrocortisone on CHX-induced suppression of β-casein mRNA accumulation in COMMA-D cells*

COMMA-D cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and with or without hydrocortisone. Cells were then cultured for 8 h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. CHX was added to the culture medium from 15 min prior to the addition of prolactin. Cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the probe used is shown on the right and the estimated size (kilobases) of the mRNA hybridising on the left.

The extracellular matrix

Interactions between mammary cells and the basement membrane are not only critical for mammary cell differentiation, but also influence the expression of milk protein genes (Li *et al.*, 1987). To test the effect of the extracellular matrix on CHX-dependent suppression of β-casein mRNA accumulation, COMMA-D cells were cultured for 2 d in the presence of insulin and hydrocortisone on either plastic, collagen- or *Matrigel®*-coated plastic. Cells were then cultured for 8 h with or without prolactin and/or CHX. β-casein mRNA accumulation was stimulated in the presence of prolactin on all cell culture matrices (**Fig. 23**, lane 3). However, the level of induction of β-casein gene expression was dependent on the extracellular matrix. β-casein mRNA accumulated to the highest levels in cells cultured on *Matrigel®*-coated plastic, but to lower levels on collagen-coated plastic and plastic alone, consistent with the results of experiments conducted by Medina *et al.*, (1987) and Eisenstein & Rosen, (1988). CHX-dependent suppression of β-casein gene expression was observed in prolactin-treated COMMA-D cells cultured on all cell culture surfaces, indicating that the effect of protein synthesis inhibition is independent of the extracellular matrix.

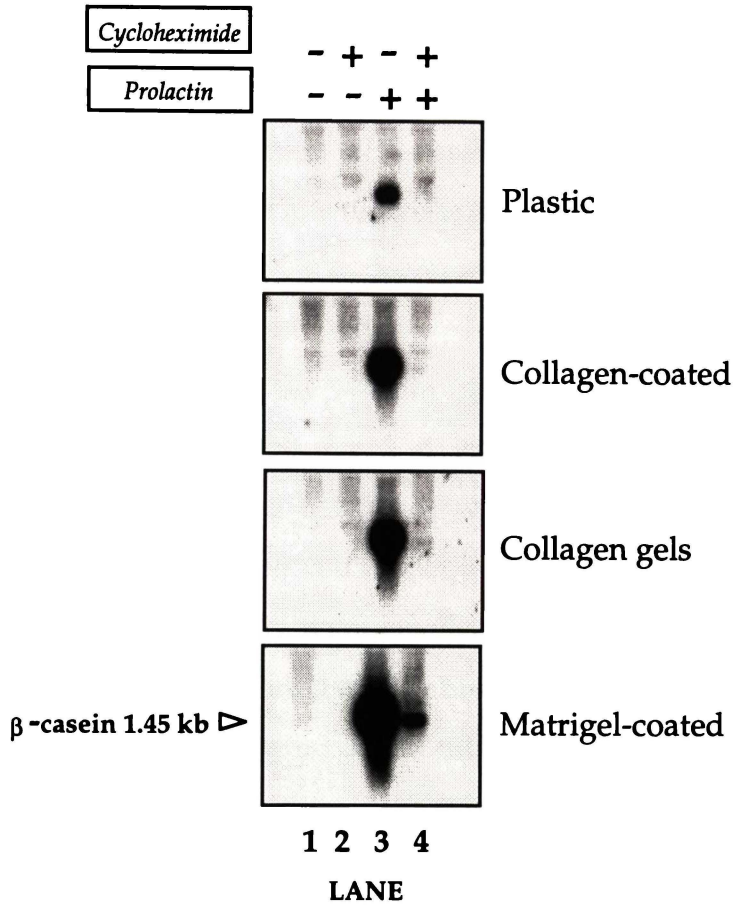


Figure 23 *Effect of extracellular matrix on CHX-dependent suppression of β-casein mRNA accumulation in COMMA-D cells*

COMMA-D cells were cultured on plastic, collagen-coated plastic, floating collagen gels or *Matrigel®*-coated plastic for 2 d in the presence of hydrocortisone and insulin, and then for 8 h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. CHX was added to the culture medium from 15 min prior to the addition of prolactin. Cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis as described in Fig. 15. For each blot, the type of extracellular matrix used is shown on the right.

(iii) How is the effect of CHX on β-casein mRNA accumulation mediated?

It has been shown that hormonal induction of β-casein gene expression not only involves the stimulation of β-casein gene transcription but also increased stability of the transcripts (Guyette *et al.*, 1979; Poyet *et al.*, 1989). It is possible that the effect of CHX is to decrease transcription, decrease mRNA stability, or a combination of these. To distinguish between these possibilities, the effect of CHX on β-casein transcription rate was examined. COMMA-D cells were cultured for 2 d in the presence of insulin and hydrocortisone, and then for 8 h with or without prolactin and/or CHX. Cells were then harvested, lysed and nuclei were isolated for nuclear run-on transcription assays. The nuclei were labelled in the presence of [α-^{32}P]UTP for 30 min, total RNA was isolated and hybridised to β-casein cDNA which had been previously UV-crosslinked to nitrocellulose membranes. Two separate experiments were conducted to examine the effect of CHX on β-casein gene transcription. However, high levels of background radioactivity bound non-specifically to the membranes prevented the determination of whether prolactin-stimulated β-casein gene transcription was altered in the presence of CHX (results not shown).

The effect of CHX on β-casein mRNA stability was also determined. Cells were cultured for 2 d in the presence of insulin and hydrocortisone, then for 8 h in the presence of prolactin and with or without CHX. Following the 8 h incubation period, the cells were cultured for a further 4 or 8 h under the same conditions, this time in the presence or absence of *5,6*-dichlorobenzimidazole riboside (DRB), an RNA synthesis inhibitor. Cells were harvested, total RNA was isolated, and equivalent amounts of RNA were subjected to electrophoresis and blotted for Northern analysis. To determine any effect of CHX on β-casein mRNA stability, β-casein mRNA levels were quantified and are shown in **Table 8** (normalised to 28S rRNA).

Table 8 *Effect of cycloheximide on the stability of β-casein transcripts*

CHX treatment	DRB treatment (time, h)	β-casein mRNA (Integrated density*)
-CHX	0	2.10 ± 0.36
	4	2.03 ± 0.31
	8	2.02 ± 0.43
+CHX	0	0.12 ± 0.005
	4	0.16 ± 0.005
	8	0.22 ± 0.005

* normalised to 28S rRNA, expressed as means of duplicate estimations ± half the range

When RNA synthesis was blocked, the levels of β-casein mRNA in prolactin-treated cells remained relatively constant after 8 h, independent of CHX treatment. This suggests that the stability of β-casein transcripts was not significantly altered when protein synthesis was inhibited for 8 h. This experiment was also repeated using another RNA synthesis inhibitor, actinomycin D. The results of this experiment also showed that the stability of β-casein transcripts was not significantly altered in the presence of CHX over 8 h (results not shown). This result has also been observed by others (Yoshimura & Oka, 1990). Together, this evidence suggests that the CHX-dependent suppression of β-casein mRNA accumulation is not the result of a decrease in the stability of β-casein gene transcripts.

Discussion

The requirement for ongoing protein synthesis for β-casein gene expression was studied in COMMA-D and primary mouse mammary epithelial cells. Hormonal induction of β-casein mRNA accumulation was significantly reduced (by approximately 95%) when protein synthesis was inhibited in COMMA-D and primary cells, suggesting that ongoing protein synthesis is required for maximal β-casein gene expression. Furthermore, protein synthesis is not only required for activation, but also for maintenance of β-casein gene expression. Together with the results of other studies (Yoshimura & Oka, 1990; Goodman & Rosen, 1990), the results presented in this chapter suggest that the effect of protein synthesis inhibition on β-casein mRNA levels is mediated via a reduction in the rate of transcription of the β-casein gene, but that the stability of the β-casein transcripts is unchanged.

Cycloheximide has a range of effects in the cell

CHX is a very potent inhibitor of protein synthesis, and has been successfully used in a number of *in vivo* and *in vitro* experiments designed to test the requirement for protein synthesis in various tissues and cell types. For example, Gronowski *et al.*, (1996) have tested the requirement for ongoing protein synthesis during growth hormone signalling in the mouse liver, illustrating how CHX can be used to delineate the rapid, primary effects of a particular hormone from secondary effects which require ongoing protein synthesis. This approach is particularly applicable to hormones such as growth hormone and prolactin, since each stimulates a number of effects in cells.

In addition to inhibiting the peptidyl transferase activity of the 60S ribosomal subunit, CHX is known to have a wide range of secondary effects in the cell. Firstly, the presence of CHX is known to lead to the superinduction, or over-accumulation of transcripts for certain genes, including the early response genes (Cochran *et al.*, 1983; Lau & Nathans,

1987). From the results of a number of experiments, it is thought that this phenomenon is the result of several contributory mechanisms. These include the loss of labile mRNA degrading enzymes (Shaw & Kamen, 1986; Greenburg *et al.*, 1986), an inability to shut off transcription of genes for which there is evidence for autorepression (Greenburg *et al.*, 1986; Fort *et al.*, 1987), and the loss of labile repressors required to maintain some genes in an inactive state (Wall *et al.*, 1986). Following the inhibition of protein synthesis, mRNA-degrading enzymes, gene products or other repressors would rapidly disappear.

CHX also has a number of specific effects. For instance, CHX is known to stimulate the rapid induction of the proto-oncogenes *c-fos* and *c-jun* (Edwards & Mahadevan, 1992), the prolonged induction of a stress-activated protein kinase, the transient induction of MAP kinase (Zinck *et al.*, 1995), and the phosphorylation of some cellular proteins (Mahadevan *et al.*, 1991). In addition, CHX and anisomycin are both capable of interacting with the intracellular signalling biochemistry of cells by stimulating the same intracellular signals as epidermal growth factor (EGF), to act as EGF agonists (Mahadevan *et al.*, 1991; Mahadevan, 1991). Thus, CHX and anisomycin can act positively to switch on transcription. Clearly, the observed reduction in β-casein mRNA accumulation is not consistent with superinduction or enhanced stability of β-casein transcripts, nor is there any evidence for autorepression of the β-casein gene. Nevertheless, the possible implications of other secondary effects of CHX in the cell should be taken into account, especially when considering how the effect of CHX on β-casein gene expression is mediated in these cells.

Characterisation of the effect of CHX on β-casein gene expression

The effect of protein synthesis inhibition on β-casein gene expression was investigated in two types of mammary epithelial cells, the COMMA-D cell line as well as primary mammary epithelial cells isolated from day 14-15 pregnant mice. A major limitation of *in vitro* experiments is that cell culture

conditions do not mimic all of the hormonal signals, cell-cell and cell-matrix interactions processed by mammary cells *in vivo*. In fact, mammary cells in culture display only some of the characteristics of lactating mammary cells. For example, COMMA-D cells are a mixture of several cell types within which only 10-20% of the cells are capable of casein synthesis in response to hormonal stimuli (Medina *et al.*, 1987). The low percentage of hormonally responsive cells in the COMMA-D cell line may be due to the absence of specific cell types normally found in the mammary gland *in vivo*, resulting in reduced cell-cell and cell-matrix interactions that are required for high level mammary function. Nevertheless, *in vitro* cell culture is a valuable tool with which to investigate mammary function in the absence of the more complex environment of the mammary gland. Not only can milk protein gene expression be induced in mammary cells in culture by the synergistic actions of insulin, hydrocortisone and prolactin, but conditions can be manipulated to investigate the effect of any one factor on milk protein gene expression.

A number of factors are known to modulate prolactin's effect on milk protein gene expression, the most well studied of which are the hormones insulin and hydrocortisone, and the extracellular matrix. The CHX-dependent suppression of β-casein mRNA accumulation was shown to be independent of all three factors, indicating that the requirement for protein synthesis is not to synthesise proteins which mediate these signals.

How is the effect of CHX on β-casein gene expression mediated in mammary cells?

As a first step in determining how the effect of CHX is mediated in mammary cells, the question of whether CHX alters β-casein gene transcription and/or mRNA stability was addressed. Goodman & Rosen, (1990) found that treatment of COMMA-D cells with CHX significantly reduced (>95%) the level of sense transcription across the β-casein gene. Furthermore, this effect was also replicated in mouse mammary explants. Yoshimura & Oka, (1990) showed that the incorporation of [5,6-^{3}H]uridine into β-casein transcripts was significantly reduced when mouse mammary

explants were cultured in the presence of both prolactin and CHX. Thus, the results of independent studies show that CHX treatment inhibits the induction of β-casein gene transcription in mouse mammary cells. In the present study, nuclear run-on assays were performed to confirm the effect of CHX on β-casein transcription in COMMA-D cells, but the results were not conclusive. However, when the effect of CHX on β-casein mRNA stability was investigated, β-casein transcripts in hormonally stimulated COMMA-D cells were shown to be very stable, independent of CHX treatment. This is consistent with the results of Yoshimura & Oka, (1990) who showed that the stability of β-casein transcripts is unchanged in mouse mammary explants cultured in the presence of CHX. By implication, it appears then that CHX reduces β-casein gene transcription, but does not alter the stability of the transcripts. Interestingly, CHX also decreased the background levels of β-casein transcripts in primary cells isolated from day 14 - 15 pregnant mice. It is thought that the β-casein transcripts present in the mammary gland at this stage are highly unstable. Thus, any further effect of CHX on the stability of these transcripts seems unlikely.

In summary, ongoing protein synthesis appears to be required for maximal β-casein gene expression in COMMA-D and primary mouse mammary epithelial cells, and it appears that this rapid effect of protein synthesis inhibition on β-casein mRNA levels is mediated via a reduction in the rate of transcription of the β-casein gene.

Chapter Five -
What is the molecular mechanism of cycloheximide action?

The question of whether ongoing protein synthesis is required to mediate lactogenic signals in mouse mammary epithelial cells was addressed in the previous chapter (Chapter Four). It is possible that the effect of CHX on β-casein gene expression may be the result of several contributory mechanisms, which if defined may improve our understanding of how lactation is regulated. Firstly, the synthesis of a positively-acting protein(s), which is not synthesised in the presence of CHX, may be required for efficient β-casein gene transcription. Secondly, inhibiting protein synthesis may either stimulate the activation, or prevent the degradation/removal of a repressor of β-casein transcription. Alternatively, CHX may inhibit β-casein gene expression as a result of one or more of the secondary effects of protein synthesis inhibitors in the cell. The experiments described in this chapter will attempt to distinguish between these possibilities, to determine the molecular basis for how the effect of CHX on β-casein gene expression is mediated in mammary cells.

Results

(i) What is the mechanism of CHX action?

Besides its potent inhibition of protein synthesis, CHX is known to have a number of secondary effects in cells, two of which may affect β-casein gene transcription. CHX stabilises certain transcripts and is also able to stimulate some of the same intracellular signals as EGF, both of which result in the activation of the transcription factor, AP-1 (Roger *et al.*, 1998). In the mouse mammary epithelial cell line, HC11, lactogenic hormone treatment leads to a consistent reduction in the AP-1 activity levels (Jehn *et al.*, 1992), and it has been suggested that the maintenance of high cellular AP-1 levels acts as a barrier to the differentiation of epithelial cells, to the activation of the β-casein promoter, or both. Thus, it is possible that high AP-1 levels inhibit maximal β-casein gene expression in CHX-treated COMMA-D cells.

To examine the effect of prolactin- and/or CHX-treatment on the AP-1 activity levels in COMMA-D cells, nuclear proteins were isolated from cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX. EMSA were performed using the AP-1 oligonucleotide as the radioactive probe. AP-1 DNA binding activity was detected in all samples, but not at the same levels (**Fig. 24**, lanes 2-5). AP-1 activity levels were not significantly altered in prolactin-treated cells, compared with untreated cells (lanes 2 & 4). However, AP-1 activity levels were increased in response to CHX-treatment (lanes 3 & 5), consistent with the results of previous studies (Roger *et al.*, 1998). Although CHX stimulates the activation of AP-1 in COMMA-D cells, there is no evidence that high levels of AP-1 exert a direct inhibitory effect on the β-casein promoter, and there are no obvious AP-1 binding elements in the proximal β-casein promoter sequence, -344 to -1 (Jehn *et al.*, 1992). Thus, there is no conclusive evidence which shows that the effect of CHX on β-casein gene expression is mediated via the maintenance of high AP-1 activity levels in COMMA-D cells. However, these results do not preclude the involvement of AP-1 through protein-protein interactions at the proximal promoter.

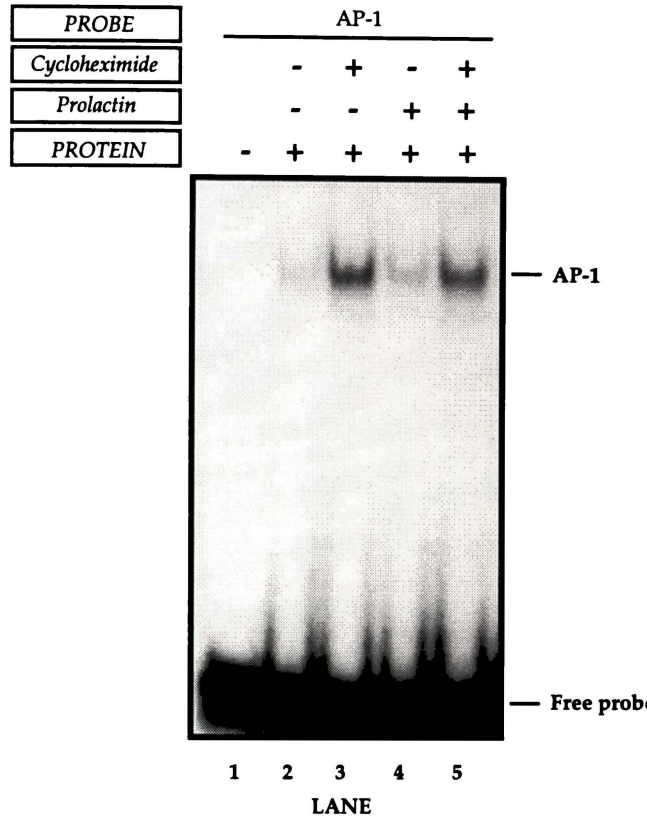


Figure 24 *Cycloheximide treatment increases the AP-1 activity levels in COMMA-D cells*

Radioactively labelled AP-1 oligonucleotide (lanes 1-5) was incubated without protein (lane 1), or with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX. Protein-DNA complexes were subject to electrophoresis in 6% non-denaturing polyacrylamide (40:1) gels as described in Materials and Methods. Free probe is indicated.

(ii) Is STAT5 protein synthesis or activation affected by CHX treatment in COMMA-D cells?

The response to prolactin is mediated, at least in part, via phosphorylation of the latent transcription factor, STAT5. STAT5 protein levels were measured by Western analysis, to determine whether CHX treatment reduces the pool of pre-existing STAT5 present in COMMA-D cells. COMMA-D cells were cultured for 2 d in the presence of insulin and hydrocortisone, and then for 8 h in the presence or absence of prolactin and/or CHX. Following hormonal stimulation, the cells were harvested and total proteins were isolated. Equivalent amounts of proteins were separated by electrophoresis in a 9% (w/v) polyacrylamide-SDS gel and transferred to nitrocellulose membrane. Two closely related proteins, called STAT5a and STAT5b, have been shown to bind to the STAT5 recognition sequence in responsive genes. Since both proteins can be detected in mammary cells (Liu *et al.*, 1996), and the functional differences between the two proteins are still to be determined, the levels of both proteins were measured on replicate blots using anti-STAT5a and anti-STAT5b antibodies, respectively (see **Appendix 1**). The levels of STAT5a or STAT5b were not significantly altered in COMMA-D cells cultured for 8 h in the presence or absence of prolactin and/or CHX (**Fig. 25**, lanes 1-12). These results show that CHX treatment does not significantly alter the levels of pre-existing STAT5a or STAT5b protein in COMMA-D cells over an 8 h time period.

Upon activation, dimerised STAT5a and STAT5b are translocated to the nucleus and bind to STAT5 consensus sequences within the promoters of target genes to activate transcription. Prolactin stimulates the rapid and transient induction of STAT5 DNA-binding activity in COS cells co-transfected with cDNA encoding STAT5 and the prolactin receptor (Gouilleux *et al.*, 1994). To confirm activation of STAT5 in COMMA-D cells, nuclear proteins were extracted from cells which were cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1, 4 or 8 h in the presence of prolactin for electrophoretic mobility shift assays (EMSA).

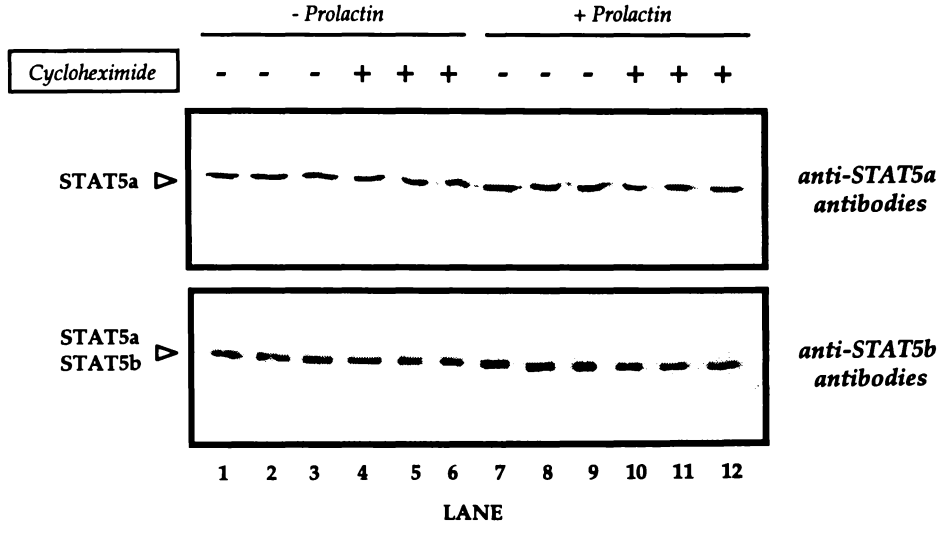
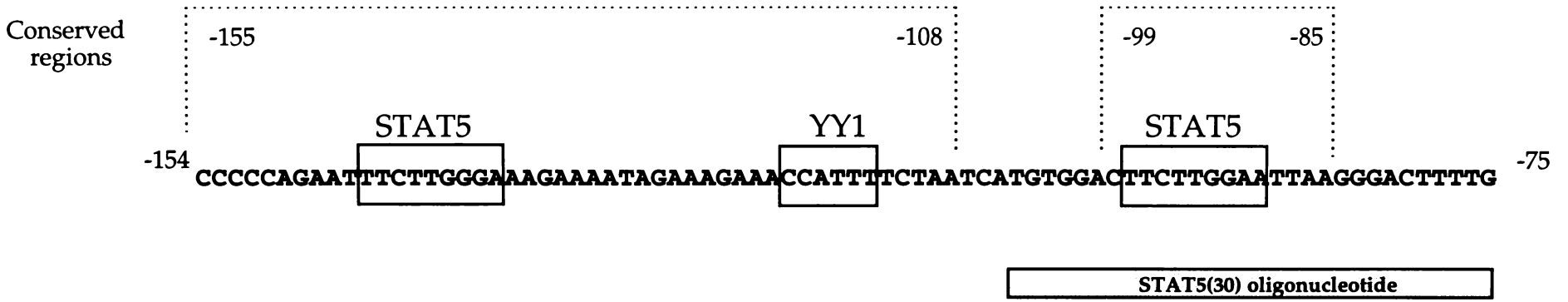


Figure 25 *STAT5a and STAT5b protein levels in COMMA-D cells*

COMMA-D cells were cultured for 2d in the presence of insulin and hydrocortisone, and then for 8h in the presence or absence of 5 μg/mL prolactin and/or 40 μg/mL CHX. Following prolactin treatment, total proteins were isolated from the cells and equivalent amounts of proteins (15 μg) were subjected to electrophoresis in a 9% (w/v) polyacrylamide gel and transferred to Polyscreen® PVDF membrane for Western analysis. The blot was probed with anti-STAT5a antibodies and the signal was detected using enhanced chemiluminescence (ECL) as described in Materials and Methods. An identical blot was probed with anti-STAT5b antibodies as described above.

A 30 bp oligonucleotide sequence derived from the rat β-casein promoter (-104 to -75), denoted STAT5(30), was used as the radioactive probe (see **Fig. 26**). STAT5 DNA-binding activity was detected in the nuclear proteins of COMMA-D cells cultured for 1 h in the presence of prolactin, but not after 4 or 8 h of prolactin treatment (results not shown). No STAT5 DNA-binding activity was detected in control cells cultured in the absence of prolactin. Thus, prolactin stimulates the rapid and transient activation of STAT5 DNA-binding activity in COMMA-D cells.

To investigate whether protein synthesis inhibition affects prolactin-stimulated STAT5 DNA-binding activity, EMSA were performed using nuclear proteins extracted from COMMA-D cells. The cells were cultured for 2 d in the presence of insulin and hydrocortisone, and then 1 h in the presence or absence of prolactin and/or CHX. Using the STAT5(30) oligonucleotide as the radioactive probe, three prominent protein complexes were detected (**Fig. 27,** labelled complexes I, II and III). Prolactin treatment induced the formation of complex I (lane 3), and the presence of STAT5 in this retarded band was confirmed using anti-STAT5a and anti-STAT5b antibodies. The addition of either anti-STAT5a or anti-STAT5b antibodies to the EMSA reaction reduced the density of complex I and initiated the formation of two 'supershifted' bands (lanes 6-7). Since anti-STAT5a antibodies are specific for STAT5a and non-crossreactive with STAT5b, and anti-STAT5b antibodies are crossreactive with STAT5b and, to a lesser extent, with STAT5a, these results confirm the presence of only STAT5a in this protein complex. Although STAT5a/STAT5b heterodimers may be present, this can not be confirmed using these antibodies. The formation of complex I/STAT5 was significantly inhibited by CHX-treatment (lane 4).



adapted from Groner & Gouilleux, 1995

Figure 26 Nuclear factor binding sites in part of the rat β-casein promoter (-154 to -75).

The binding sites for nuclear factors which are known to bind to this region of the β-casein promoter are boxed. Two binding sites for STAT5 have been identified. A low affinity site is present between positions -136 and -146 and a high affinity site is present between positions -89 and -99. The recognition sequence for YY1 is present between positions -113 and -118. The regions between -85 and -99, and -108 and -155 are strongly conserved in the casein genes of several species. The STAT5(30) oligonucleotide (corresponding to nucleotides -104 to -75) contains the high affinity STAT5 binding site.

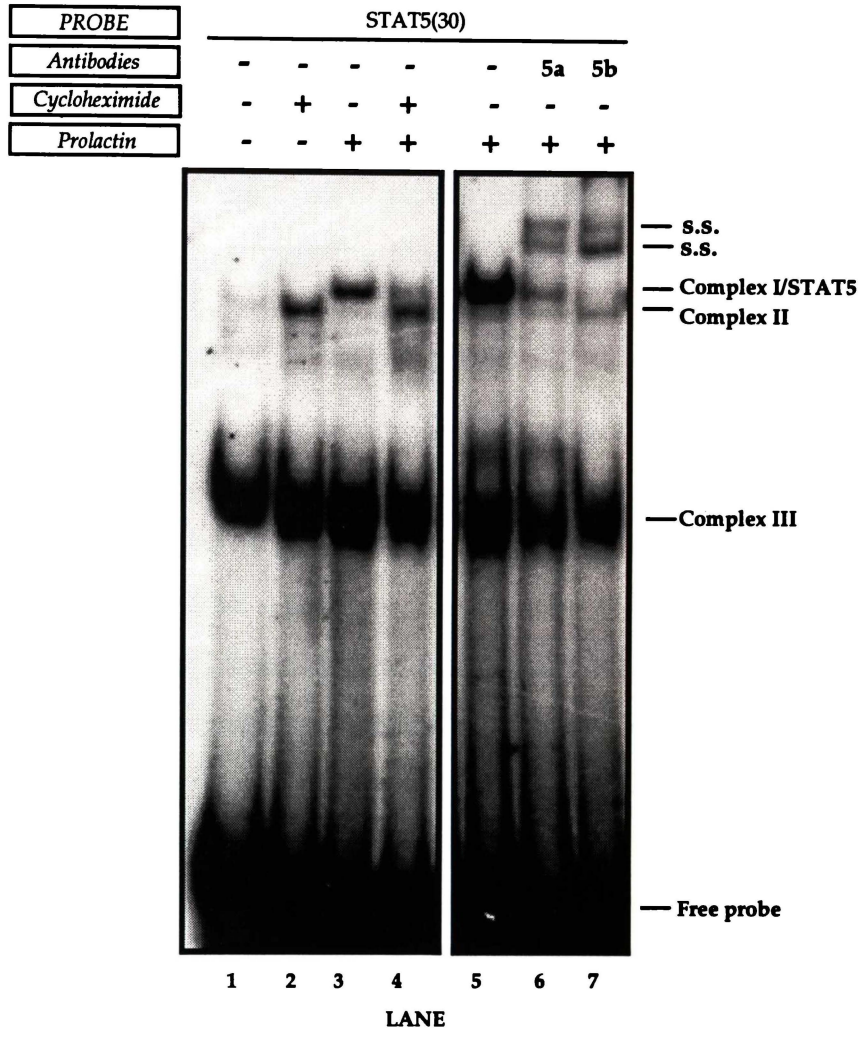


Figure 27 *Cycloheximide stimulates the activation of a DNA-binding activity (complex II), of distinct mobility than that containing STAT5 (complex I).*
Radioactively labelled STAT5(30) oligonucleotide (lanes 1-7) was incubated with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 1 h in the presence or absence of prolactin and/or CHX (lanes 1-4). In addition, the STAT5(30) probe was incubated with 10 μg nuclear proteins isolated from COMMA-D cells cultured for 1 h in the presence of prolactin (lane 5), and either 1 μg of anti-STAT5a antibodies (lane 6) or 1 μg of anti-STAT5b antibodies (lane 7). Protein-DNA complexes were analysed as described in Fig. 24. Free probe is indicated. s.s., 'supershifted' band; 5a, anti-STAT5a antibodies; 5b, anti-STAT5b antibodies.

In addition, CHX-treatment induced the formation of a second complex (complex II), of different mobility than that identified as containing STAT5 (**Fig. 27**, lanes 2 & 4). Co-stimulation of COMMA-D cells with prolactin and CHX induced the formation of both complex I/STAT5 and complex II (lane 4). Of note, no additional complex of slower mobility was observed in prolactin- and CHX-treated cells, suggesting that STAT5 and a second protein (complex II) bind to the STAT5(30) probe in a mutually exclusive manner.

A broad, diffuse band (complex III) was observed in all reactions containing nuclear proteins derived from COMMA-D cells (**Fig. 27**, lanes 1-7). Similar lower mobility bands have also been observed by others (Schmitt-Ney *et al.*, 1992a; 1992b). However, the identity of the protein(s) within this complex is currently unknown and no attempt was made to further clarify this. Nevertheless, excess unlabelled STAT5(30) oligonucleotide competed with all three protein complexes (complexes I, II & III, **Fig. 28,** lanes 3-4 & 7-8). Addition of excess unlabelled oligonucleotide containing a DNA-binding consensus sequence for Sp1 had no effect on the complexes (lanes 5 & 9), verifying the binding specificity of the proteins to the STAT5(30) probe. These results show that three distinct protein complexes associate with the STAT5(30) probe in prolactin- and CHX-treated cells, and that CHX treatment is associated with the formation of complex II.

To determine whether the effect of CHX on β-casein gene expression is dependent on STAT5b, mammary epithelial cells were isolated from d 14 pregnant STAT5b$^{-/-}$ mice (Udy *et al.*, 1997). Immediately following isolation, the cells were cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX. Following prolactin stimulation, the cells were harvested and total RNA was isolated for Northern analysis. The addition of prolactin to the culture medium resulted in a significant increase in β-casein mRNA levels, compared with the background levels of β-casein transcripts detected in cells isolated from the pregnant gland (**Fig. 29**, compare lanes 1 & 3).

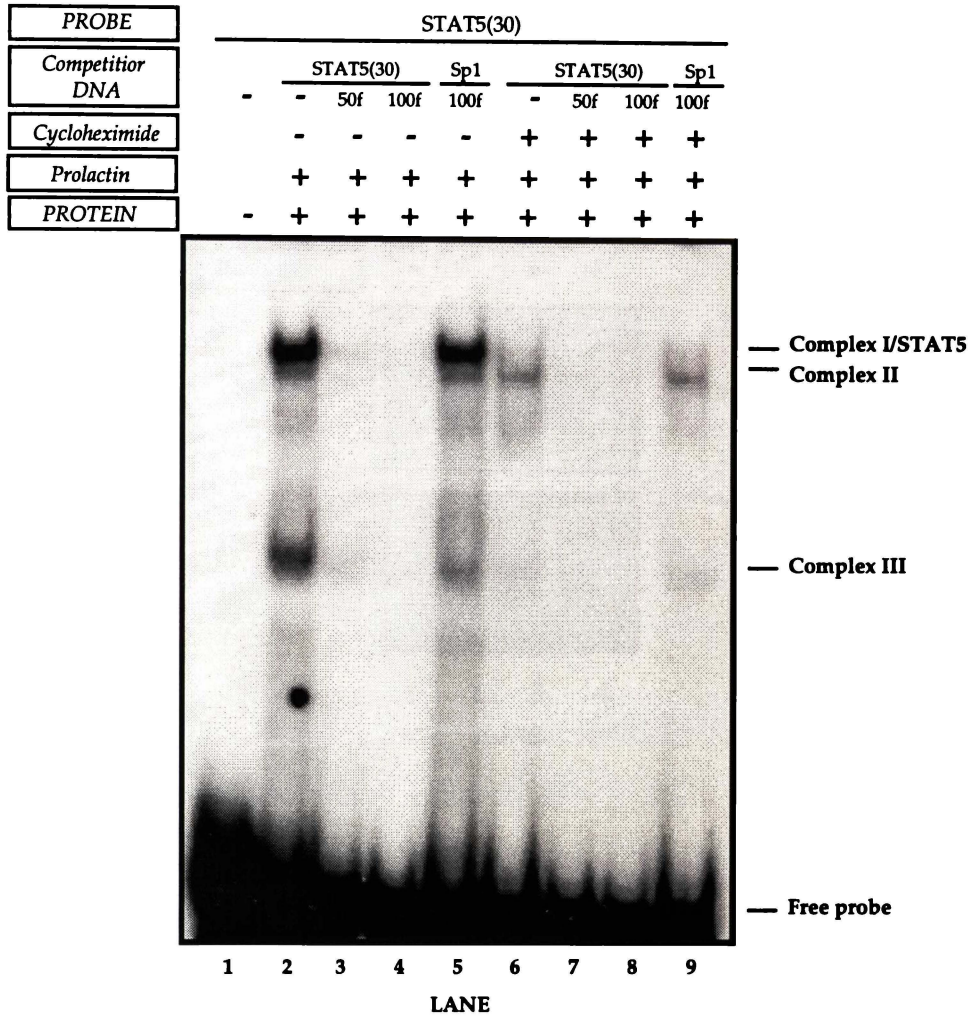


Figure 28 *Excess unlabelled STAT5(30) oligonucleotide competes with complex I, complex II and complex III for binding to the STAT5(30) probe*

Radioactively labelled STAT5(30) oligonucleotide (lanes 1-9) was incubated without protein (lane 1), or with 5µg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence of prolactin (lanes 2-5), or prolactin and CHX (lanes 6-9). In some cases, either 50-fold (lanes 3 & 7), or 100-fold molar excess (lanes 4 & 8) of unlabelled STAT5(30) oligonucleotide, or 100-fold molar excess of unlabelled Sp1 oligonucleotide (lanes 5 & 9) was added to each EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. 50f, 50-fold; 100f, 100-fold.

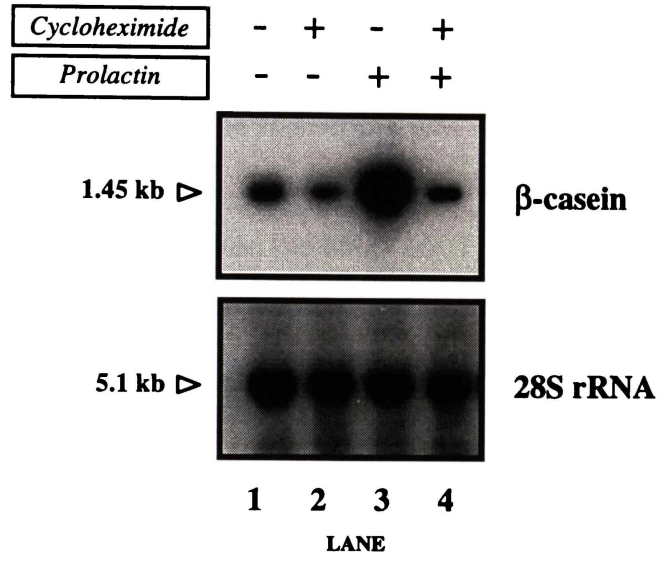


Figure 29 *Effect of CHX on the induction of β-casein mRNA accumulation in primary mammary epithelial cells isolated from STAT5b$^{-/-}$ mice*

Primary mouse mammary epithelial cells were cultured on *Matrigel®*-coated plastic for 2 d in the presence of insulin and hydrocortisone, and then for 8 h in the presence of prolactin and/or CHX. CHX was added to the culture medium 15 min prior to the addition of prolactin. Cells were harvested, total RNA was isolated, equivalent amounts of RNA (5 μg) were subjected to electrophoresis and blotted for Northern analysis. For each blot, the probe used is shown on the right and the estimated size of mRNA hybridising (kilobases) on the left.

This result is consistent with a previous study which examined the expression of the milk protein genes in mammary tissue derived from STAT5b$^{-/-}$ mice (Davey *et al.*, 1996). When primary cells were cultured in the presence of both prolactin and cycloheximide, the accumulation of β-casein mRNA was substantially reduced, compared with prolactin alone (**Fig. 29**, lanes 3-4). A CHX-dependent decrease in the background levels of β-casein transcripts was detected in cells cultured in the absence of prolactin (lanes 1-2). These results show that the effect of CHX is not dependent on STAT5b. However, the effect of CHX on β-casein gene expression may be mediated via STAT5a. STAT5a$^{-/-}$ or STAT5a$^{-/-}$5b$^{-/-}$ mice were unavailable to test this hypothesis using primary cell culture. Thus, the effect of CHX on STAT5 DNA-binding activity was investigated further in COMMA-D cells.

EGF treatment is known to inhibit the maximal accumulation of β-casein transcripts in mouse mammary epithelial cells *in vitro* (Taketani & Oka, 1983). Since CHX is capable of stimulating the same intracellular signals as EGF, it is possible that CHX inhibits β-casein gene expression by the same mechanism. One of the effects of EGF in the cell is to stimulate the activation of AP-1, which has already been addressed, however the exact mechanism of EGF action on β-casein gene expression remains unclear. Thus, EMSA were performed to investigate the effect of EGF treatment on STAT5 activity levels, and to determine whether EGF stimulated the activation of complex II. Nuclear extracts were isolated from cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX. In addition, cells were cultured in the presence or absence of EGF from d 0 (plating) or from 15 minutes prior to the addition of prolactin on day 3 of culture. STAT5 DNA-binding activity levels were slightly reduced in prolactin-treated cells cultured from d 0 in the presence of EGF, compared with cells cultured in the absence of EGF (**Fig. 30**, compare lanes 4-5). EGF treatment did not stimulate the formation of complex II (lanes 5 & 7). This is consistent with the results of other studies which suggest that EGF may inhibit β-casein mRNA accumulation via indirect effects on prolactin signalling pathways and differentiation state (Welte *et al.*, 1994). These results suggest that CHX does not inhibit β-casein gene expression via the EGF signalling pathway.

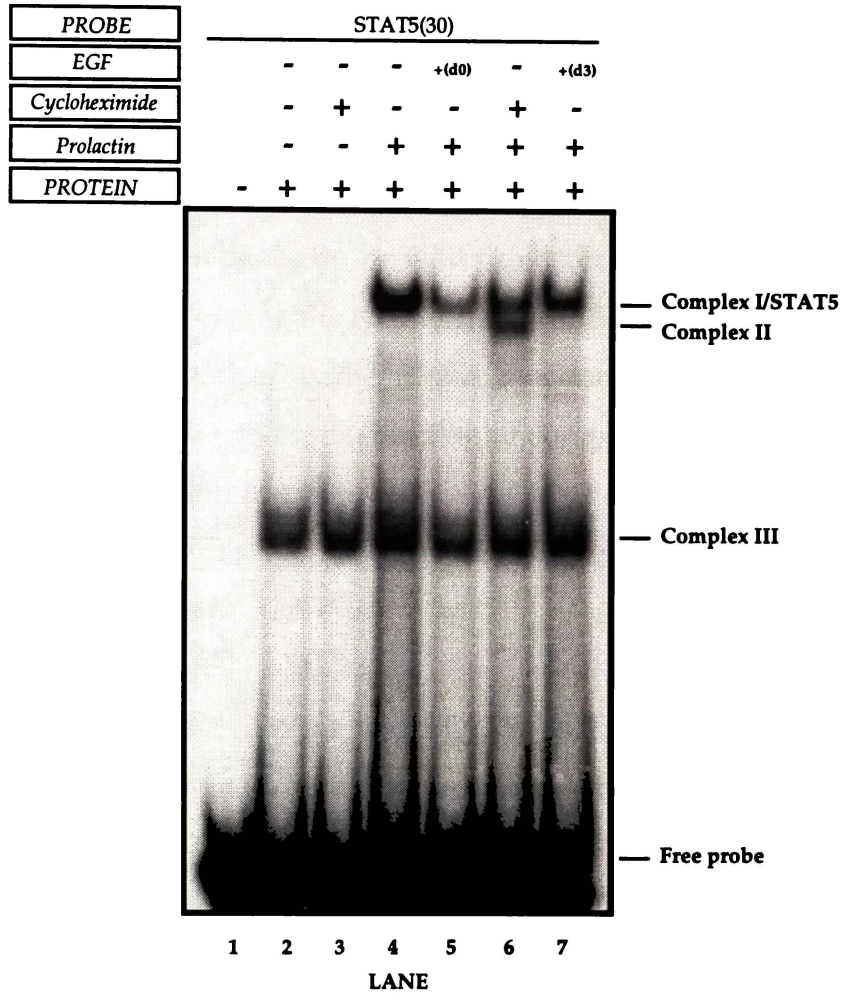


Figure 30 *EGF does not stimulate the activation of complex II in COMMA-D cells* Radioactively labelled STAT5(30) oligonucleotide (lanes 1-7) was incubated without protein (lane 1), or with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX. Cells were also cultured in the presence or absence of epidermal growth factor (EGF) from d 0 (plating) or from 15 min prior to the addition of prolactin on d 3 of culture. Protein-DNA complexes were analysed as described in Fig. 24.

(iii) Characterisation of Complex II-

To determine whether complex II contained STAT5a and/or STAT5b proteins, EMSA were performed using anti-STAT5a and anti-STAT5b antibodies, respectively. Nuclear proteins were isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, then stimulated for 1 h with prolactin, or with prolactin and CHX. The STAT5(30) oligonucleotide was used as the radioactive probe. Addition of anti-STAT5a or anti-STAT5b antibodies to the EMSA reaction 'supershifted' complex I (**Fig. 31**, lanes 1-3). However, the binding of complex II to the probe was not affected by the addition of either anti-STAT5a or anti-STAT5b antibodies to the EMSA reaction (lanes 4-6). These results suggest that complex II does not contain either STAT5a or STAT5b.

In addition to the STAT5a and STAT5b isoforms, alternatively spliced forms of STAT5 have been recently identified, which result in C-terminally truncated forms of STAT5 (Moriggl *et al.*, 1996; Wang *et al.*, 1996). STAT5 deletion mutants retain the DNA-binding motif but lack the transactivating domain, and appear to act as dominant negative inhibitors of STAT5 mediated transactivation in COS7 cells (Moriggl *et al.*, 1996). It is conceivable that the binding of a truncated form of STAT5 to the β-casein promoter could result in a dramatic reduction in β-casein mRNA accumulation in CHX-treated COMMA-D cells. Since the anti-STAT5a and anti-STAT5b antibodies used in the previous experiments were raised against carboxy-terminal regions of either STAT5a or STAT5b, C-terminally truncated forms of STAT5 will not be recognised by these antibodies. To determine whether complex II contained alternatively spliced forms of STAT5a and/or STAT5b proteins, EMSA were performed using antibodies raised against the amino-terminal region of the STAT5 protein (denoted anti-STAT5N antibodies in the text). Since STAT5a and STAT5b share 96% identity differing mainly at the carboxy-terminus of the protein, these antibodies should recognise both STAT5a and STAT5b proteins. In addition, these antibodies should also recognise C-terminally truncated forms of STAT5.

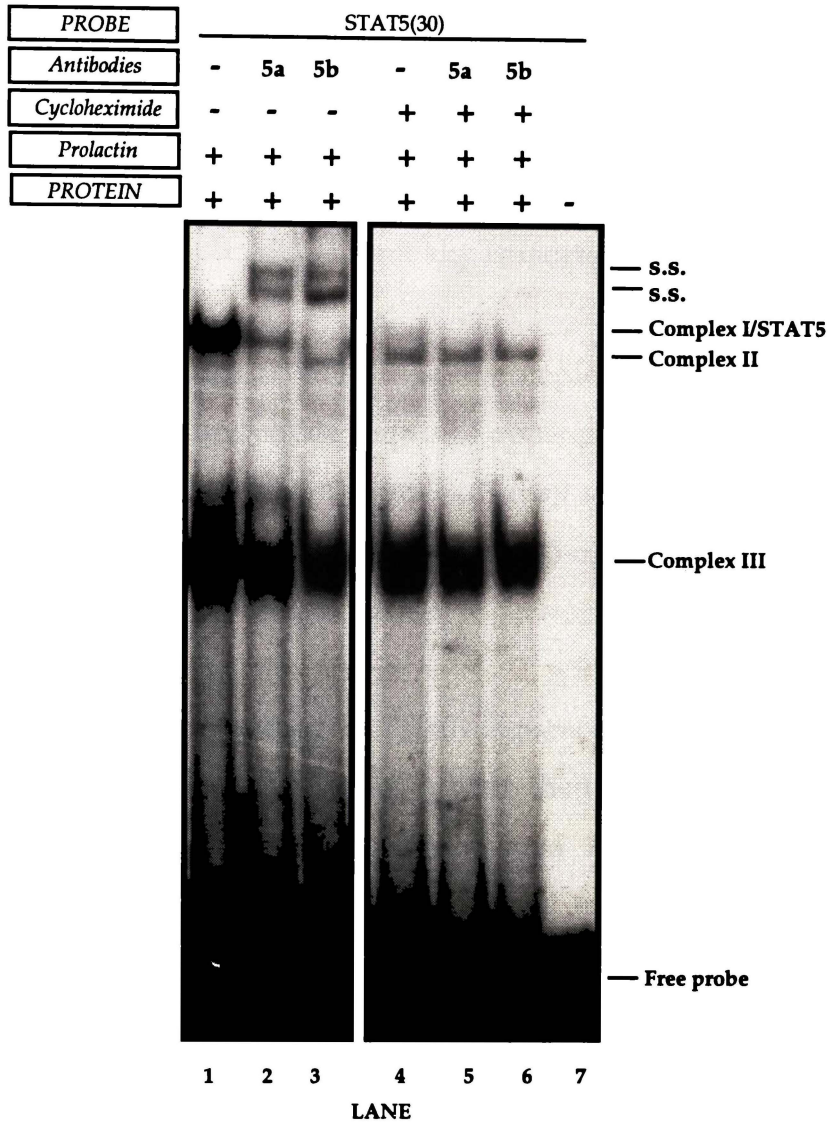


Figure 31 *Complex II does not contain immunoreactive STAT5a or STAT5b*
Radioactively labelled STAT5(30) oligonucleotide was incubated without protein (lane 7), or with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then cultured for 1 h in the presence of prolactin (lanes 1-3), or prolactin and CHX (lanes 4-6). In some cases, either 1 μg of anti-STAT5a antibodies (lanes 2 & 5), or 1 μg of anti-STAT5b antibodies (lanes 3 & 6) was added to the EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. s.s., 'supershifted' band; 5a, anti-STAT5a antibodies; 5b, anti-STAT5b antibodies. Lanes 1-3 are reproduced from Fig. 27.

The addition of anti-STAT5N antibodies to the EMSA reaction 'supershifted' complex I (**Fig. 32**, lane 4), but binding of complex II to the STAT5(30) probe was unaffected (lane 2). These results suggest that complex II does not contain alternatively spliced, C-terminally truncated forms of STAT5.

Since the binding of STAT5 and complex II to the rat β-casein promoter/STAT5(30) nucleotide sequence appears to be mutually exclusive, it is possible that the binding of complex II may sterically hinder binding of STAT5, and therefore transactivation of β-casein gene transcription. Complex II was further characterised to investigate this possibility. To characterise the binding site for complex II within the STAT5(30) probe, oligonucleotides spanning the STAT5(30) sequence were designed for use as radioactive probes (see **Fig. 33**). EMSA were then performed to examine the binding of the prolactin- and CHX-activated nuclear proteins to these sequences. The radioactive DNA probes used for these experiments were STAT5(30), STAT5(21), STAT5(30-A) and STAT5(30-B). Since all of the oligonucleotides span the STAT5 DNA-binding sequence, complex I/STAT5 bound to all of the probes (**Fig. 34**, lanes 3, 7, 11 & 15). Of note, complex I/STAT5 bound weakly to the STAT5(30-A) probe (lanes 11 & 12), suggesting that the presence of greater than two nucleotides immediately 3' to the consensus sequence (TTCNNNGAA) are required for efficient STAT5 binding. In contrast, complex II bound to the STAT5(30) and STAT5(30-B) probes (lanes 4 & 16), but did not bind to the STAT5(21) and STAT5(30-A) probes (lanes 8 & 12). These results show that complex II binds to an overlapping/adjacent site 3' to the STAT5 DNA-binding consensus sequence (TTCNNNGAA). Since complex II does not bind to the STAT5 DNA-binding consensus sequence, these results also confirm the findings of the previous experiments, which suggested that complex II does not contain STAT5.

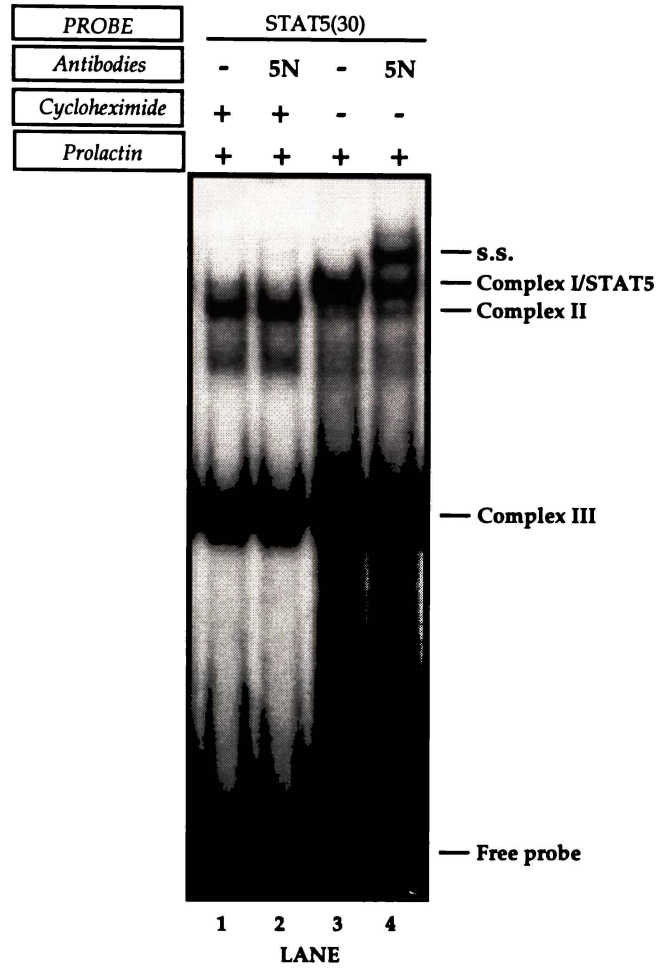


Figure 32 *Complex II does not contain an immunoreactive C-terminally truncated form of STAT5*

Radioactively labelled STAT5(30) oligonucleotide was incubated with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then cultured for 1 h in the presence of prolactin and CHX (lanes 1-2), or prolactin alone (lanes 3-4). In some cases, 1 μg of anti-STAT5N antibodies (lanes 2 & 4) was added to the EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. s.s., 'supershifted' band; 5N, anti-STAT5N antibodies.

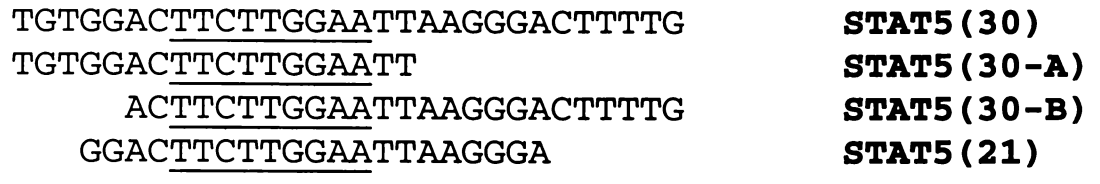


Figure 33 *Sequence comparison of the STAT5 oligonucleotides used in binding studies*

The STAT5(30) oligonucleotide corresponds to nucleotides -104 to -75 of the rat β-casein promoter sequence (Schmitt-Ney *et al.*, 1992a). The STAT5 consensus sequence is underlined. The STAT5(30-A), STAT5(30-B) and STAT5(21) oligonucleotides span the STAT5(30) sequence.

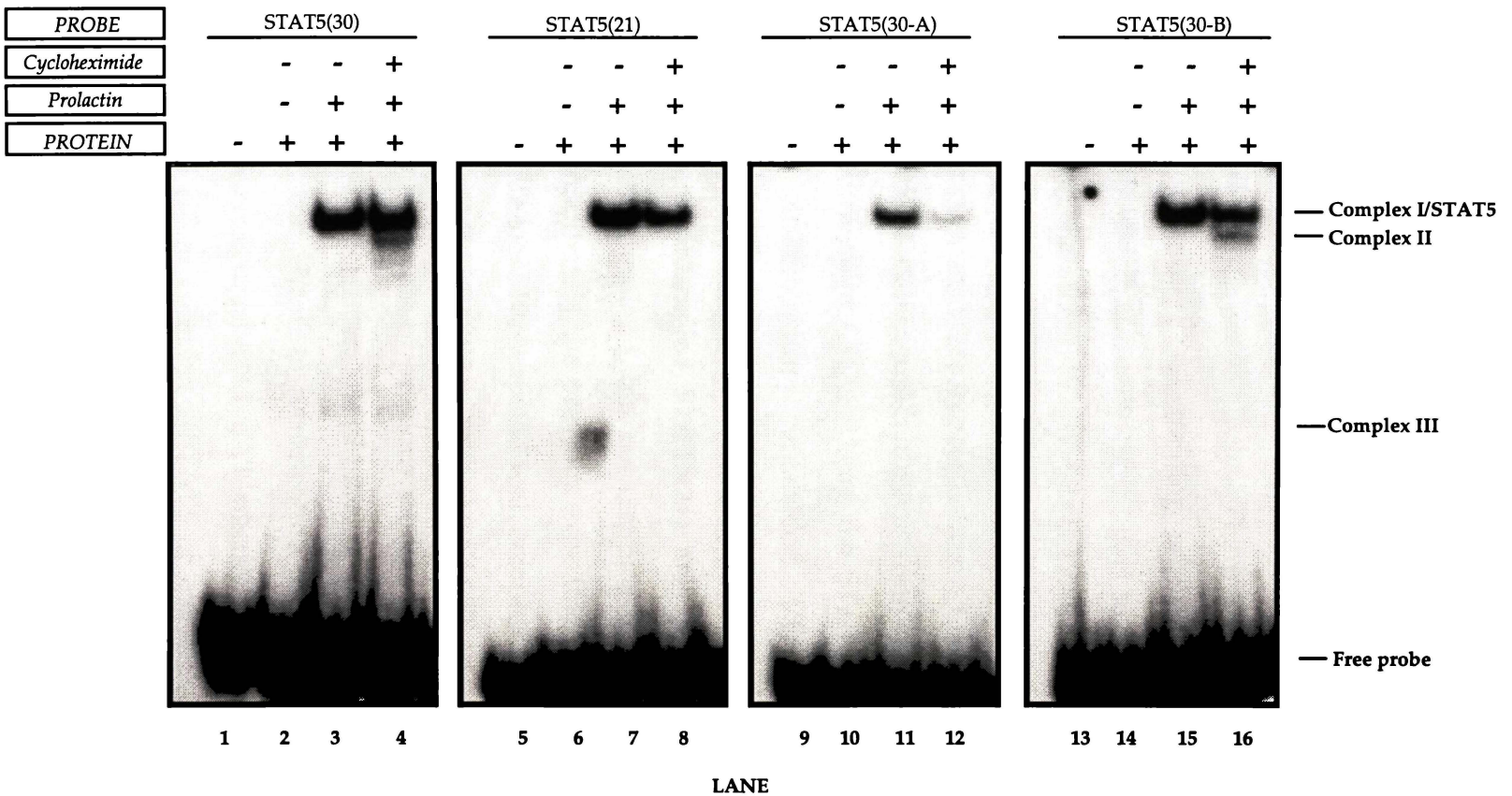


Figure 34 *Complex II does not bind to the STAT5 DNA-binding consensus sequence*
Radioactively labelled STAT5(30) oligonucleotide (lanes 1-4), STAT5(21) oligonucleotide (lanes 5-8), STAT5(30-A) oligonucleotide (lanes 9-12) or STAT5(30-B) oligonucleotide (lanes 13-16) was incubated with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 1 h in the presence or absence of prolactin and/or CHX. Protein-DNA complexes were analysed as described in Fig. 24. Free probe is indicated.

(iv) Identification of the proteins contained in Complex II-

To further analyse the protein complex induced by CHX, competition EMSA were performed. The unlabelled oligonucleotides used for these experiments were STAT5(30), STAT5(21), STAT5(30-A), STAT5(30-B), m67 SIE, YY1 and NF-κB (see **Fig. 33** and **Table 5**). As described earlier, the STAT5 oligonucleotides span the original STAT5(30) nucleotide sequence. The YY1 and NF-κB oligonucleotides contain a consensus YY1 and NF-κB recognition sequence, respectively. The m67 SIE oligonucleotide sequence contains a canonical STAT binding sequence, called the c-*sis*-inducible element (SIE), which normally contributes to the induction of c-*fos* by c-*sis* (PDGF) (Hayes *et al.*, 1987; Wagner *et al.*, 1990).

Nuclear proteins were extracted from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone and then co-stimulated for 1 h with prolactin and/or CHX. Using the STAT5(30) oligonucleotide as the radioactive probe, excess unlabelled STAT5(30) and STAT5(30-B) oligonucleotides competed with complex I and complex II (**Fig. 35**, lanes 3 & 6), and excess unlabelled STAT5(21) and STAT5(30-A) oligonucleotides competed with complex I, but not complex II (lanes 4 & 5). These results confirm the findings of the previous experiment. Excess unlabelled m67 SIE oligonucleotide weakly competed with complex I (lane 7), indicating that this probe shares some homology with the STAT5 consensus sequence. This is consistent with results of previous studies which have shown that STAT1, STAT3 and STAT5 are capable of binding to the m67 SIE probe (Sadowski *et al.*, 1983). The m67 SIE oligonucleotide did not compete with complex II (lane 7). Excess unlabelled YY1 oligonucleotide had no effect on the formation of either complex I/STAT5 or complex II (lane 8). Finally, the addition of excess unlabelled NF-κB oligonucleotide did not compete with complex I (lane 9). However, excess unlabelled NF-κB oligonucleotide competed with complex II (lane 9), showing that complex II contained the transcription factor, NF-κB.

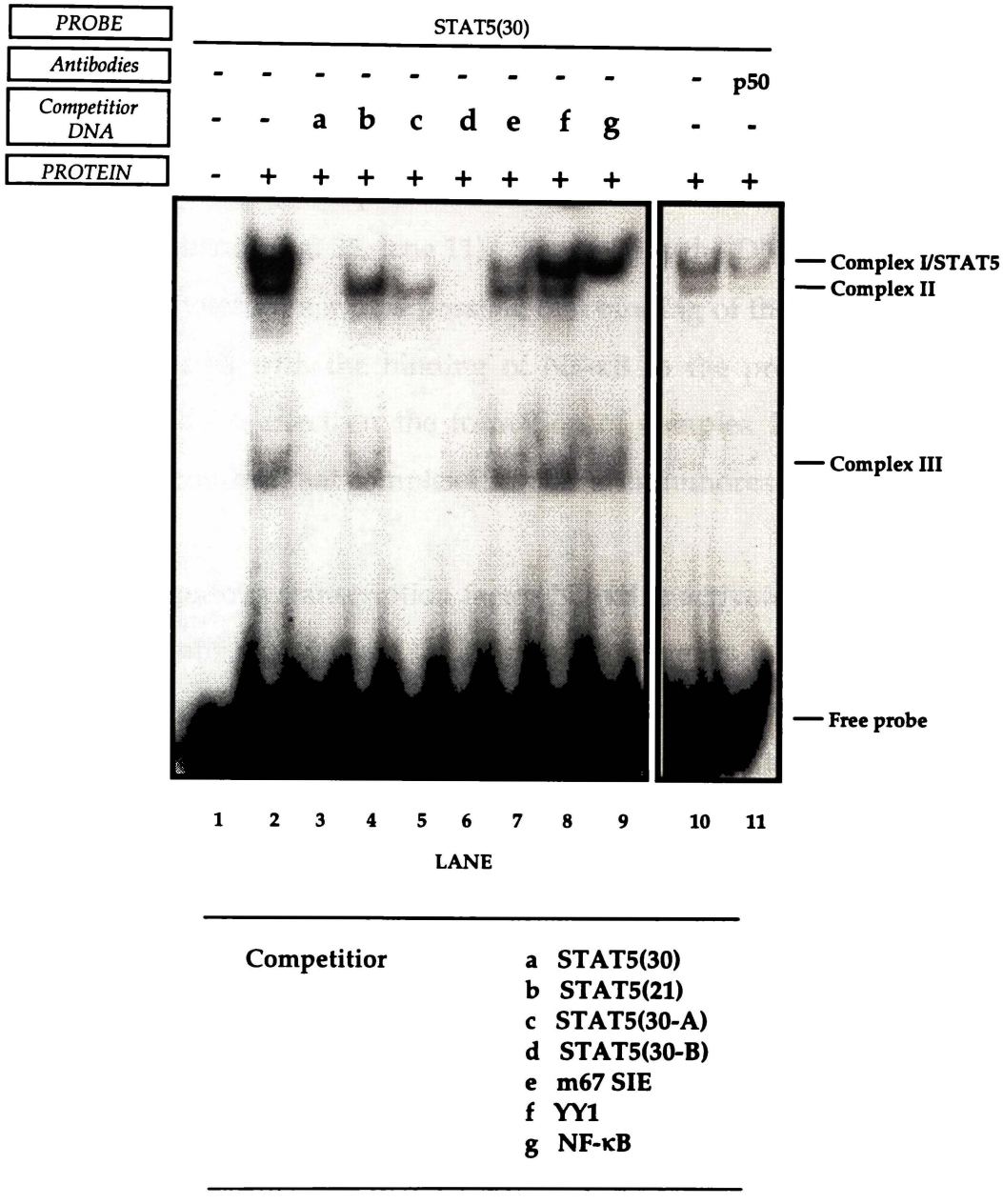


Figure 35 *Complex II contains the transcription factor, NF-κB*

Radioactively labelled STAT5(30) oligonucleotide (lanes 1-11) was incubated without protein (lane 1), or with 5μg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence of prolactin and CHX (lanes 2-11). In some cases, either 100-fold molar excess of unlabelled STAT5(30) (lane 3), STAT5(21) (lane 4), STAT5(30-A) (lane 5), STAT(30-B) (lane 6), m67 SIE (lane 7), YY1 (lane 8) or NF-κB oligonucleotide (lane 9) was added to each EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. p50, antibodies raised against the p50 subunit of NF-κB.

To confirm this result, complex II was analysed for immunoreactive NF-κB using antibodies raised against the p50 subunit of NF-κB (see **Appendix 1**). Addition of the anti-p50 antibodies to the EMSA reaction resulted in a loss of complex II, but did not initiate the formation of a 'supershifted' band (**Fig. 35**, lane 11). Since p50 is the DNA-binding subunit of the NF-κB heterodimer, it is possible that binding of the antibodies to this subunit interferes with the binding of NF-κB to the probe. The anti-p50 antibodies had no effect on the formation of complex I/STAT5 (lane 11). These results confirm that complex II contains immunoreactive NF-κB.

The ubiquitous transcription factor NF-κB is activated in response to a variety of stimuli, to regulate the expression of genes involved in immune function, the inflammatory response, cell growth and apoptosis (for a review see Baldwin, 1996). To determine whether CHX stimulates the activation of NF-κB, EMSA were performed using nuclear extracts isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX. Using the NF-κB oligonucleotide as the radioactive probe, three prominent protein complexes were detected in nuclear extracts isolated from control COMMA-D cells (**Fig. 36**, lane 2). Prolactin treatment alone had no effect on the formation of these complexes (compare lanes 2 & 4). However, CHX treatment induced the formation of an additional protein complex (arrowed in lanes 3 & 5), independent of prolactin treatment. This complex has a similar mobility to the CHX-stimulated complex II/NF-κB DNA-binding activity, which binds to the STAT5(30) probe (compare lanes 3, 5, 8 & 10). The identity of this arrowed complex was confirmed using anti-p50 antibodies (**Fig. 36**, lane 12). Addition of the anti-p50 antibodies 'supershifted' the arrowed complex, showing that it contained immunoreactive NF-κB. Thus, CHX stimulates the activation of NF-κB. The other complexes detected in control COMMA-D cells were unaffected by the addition of anti-p50 antibodies.

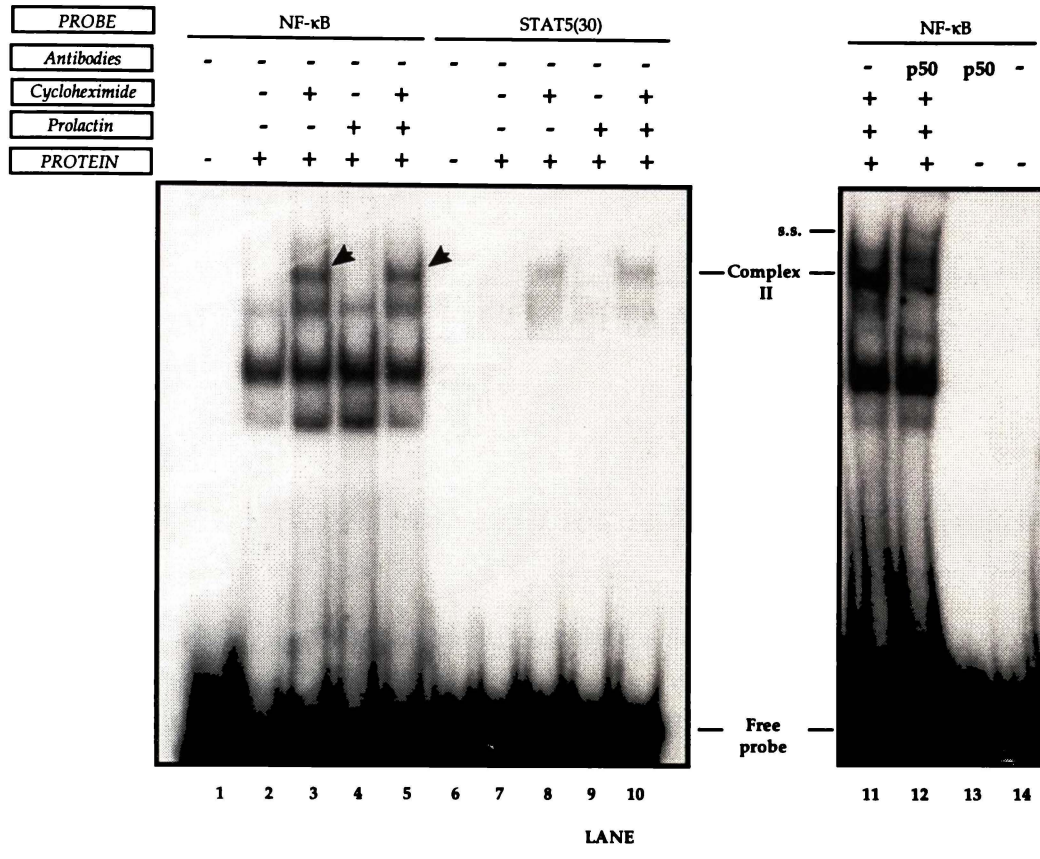


Figure 36 *Cycloheximide treatment stimulates the activation of NF-κB*

Radioactively labelled NF-κB (lanes 1-5 & 11-14) or STAT5(30) oligonucleotide (lanes 6-10) was incubated without protein (lanes 1 & 6, respectively), or with 5 μg of nuclear proteins isolated from COMMA-D cells cultured for 2 d in the presence of insulin and hydrocortisone, and then for 1 h in the presence or absence of prolactin and/or CHX (lanes 2-5 & 7-10). In addition, the NF-κB probe was incubated with nuclear proteins isolated from COMMA-D cells cultured for 1 h in the presence of prolactin and CHX (lanes 11-12), and 1 μg of anti-p50 antibodies (lane 12). Protein-DNA complexes were analysed as described in Fig. 24. Free probe is indicated. p50, anti-p50 antibodies.

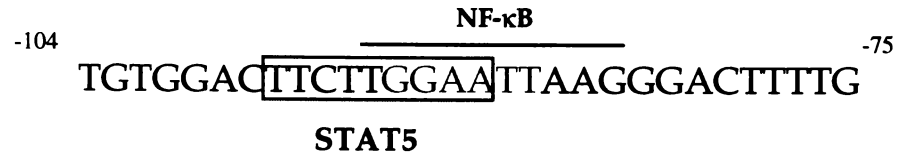


Figure 37 *Rat β-casein promoter (-104 to -75)/STAT5(30) oligonucleotide sequence*

The STAT5 DNA-binding consensus sequence is boxed in blue and a putative NF-κB element is also indicated. Nucleotides which are identical to the NF-κB DNA-binding consensus sequence are coloured in red.

This suggests that these protein complexes do not contain the p50 subunit of NF-κB, however they may contain other non-crossreactive NF-κB subunits. Further investigations would be required to verify this.

Inspection of the STAT5(30) oligonucleotide/rat β-casein promoter nucleotide sequence revealed a putative κB binding element which overlaps the STAT5 DNA-binding consensus sequence (**Fig. 37**). NF-κB is known to bind to the recognition sequence GGGAATTCCC. However, it has recently been reported that activated NF-κB factors can also associate with a κB half-site, GGGAAT (Murphy *et al.*, 1995). Thus, CHX-stimulated NF-κB is most likely to associate with the STAT5(30)/rat β-casein promoter sequence between positions -92 and -87 (GGAATT). This element overlaps the high-affinity STAT5 site, consistent with the idea of mutually exclusive binding of STAT5 and NF-κB.

(v) Is Complex II/NF-κB detectable in vivo?

To investigate whether NF-κB competes with STAT5 for binding to the STAT5(30)/rat β-casein promoter sequence *in vivo*, nuclear extracts were isolated from mammary tissue derived from day 1 lactating mice. Nuclear proteins were isolated for EMSA by either of two commonly used methods, high salt nuclear extraction (Dignam, 1983), or ammonium sulphate precipitation (Schmitt-Ney *et al.*, 1991). Using the STAT5(30) oligonucleotide as the radioactive probe, one prominent protein complex was detected in high salt extracted nuclear proteins (**Fig. 38**, lane 4). The addition of either anti-STAT5a or anti-STAT5b antibodies reduced the density of this complex and initiated the formation of a 'supershifted' band, confirming that this complex contained STAT5 (lanes 5 & 6). Complex II/NF-κB DNA binding activity was not detected in these extracts.

Of note, the formation of a distinct complex of different mobility than that identified as containing STAT5 was detected in ammonium sulphate precipitated nuclear proteins (**Fig. 38**, lane 1). This DNA-binding activity was denoted complex *A*. The binding of complex *A* to the probe was not affected by the addition of either anti-STAT5a or anti-STAT5b antibodies to the EMSA reaction (lanes 1-3). However, excess unlabelled STAT5(30) oligonucleotides competed with complex *A* (**Fig. 39**, lanes 2-3), and addition of excess unlabelled oligonucleotide containing an Sp1 recognition sequence had no effect on the formation of complex *A* (lane 4), verifying the binding specificity of this protein to the STAT5(30) probe.

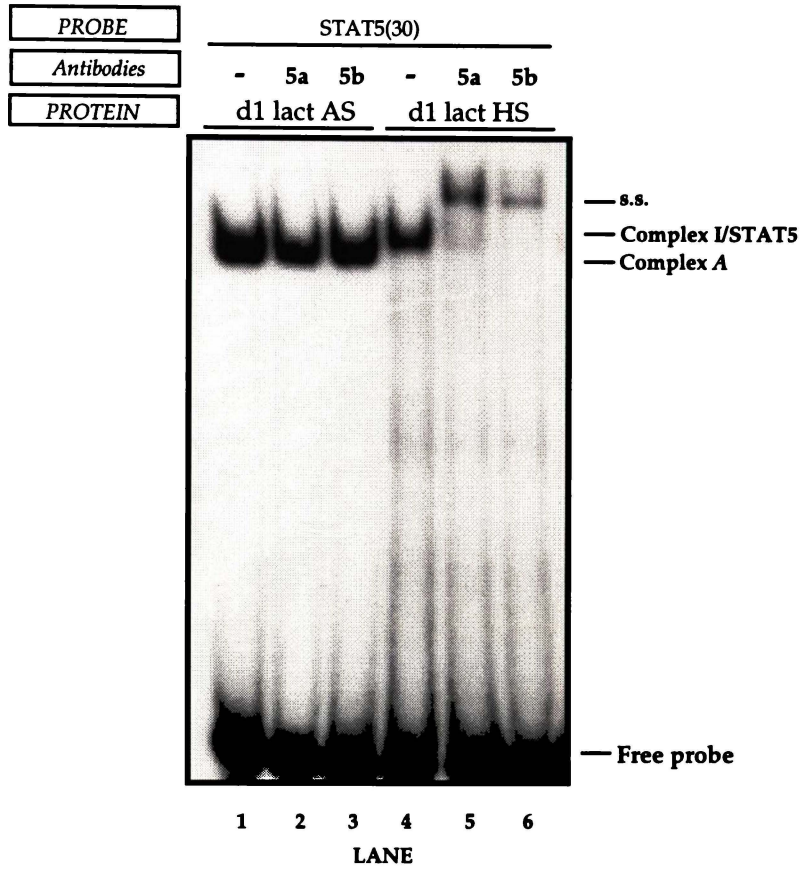


Figure 38 *A protein of distinct mobility to STAT5 and similar mobility to complex II/NF-κB was detected in ammonium sulphate precipitated mammary nuclear proteins derived from d1 lactating mice*

Radioactively labelled STAT5(30) oligonucleotide (lanes 1-6) was incubated with 5μg of mammary nuclear proteins derived from d 1 lactating mice. Nuclear proteins were isolated by either high salt extraction (lanes 4-6), or ammonium precipitation (lanes 1-3). In some cases, either 1 μg of anti-STAT5a antibodies (lanes 2 & 5) or 1 μg of anti-STAT5b antibodies (lanes 3 & 6) was added to each EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. s.s., 'supershifted' band; 5a, anti-STAT5a antibodies; 5b, anti-STAT5b antibodies; d1 lact AS, ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice; d1 lact HS, high salt extracted mammary nuclear proteins derived from d 1 lactating mice.

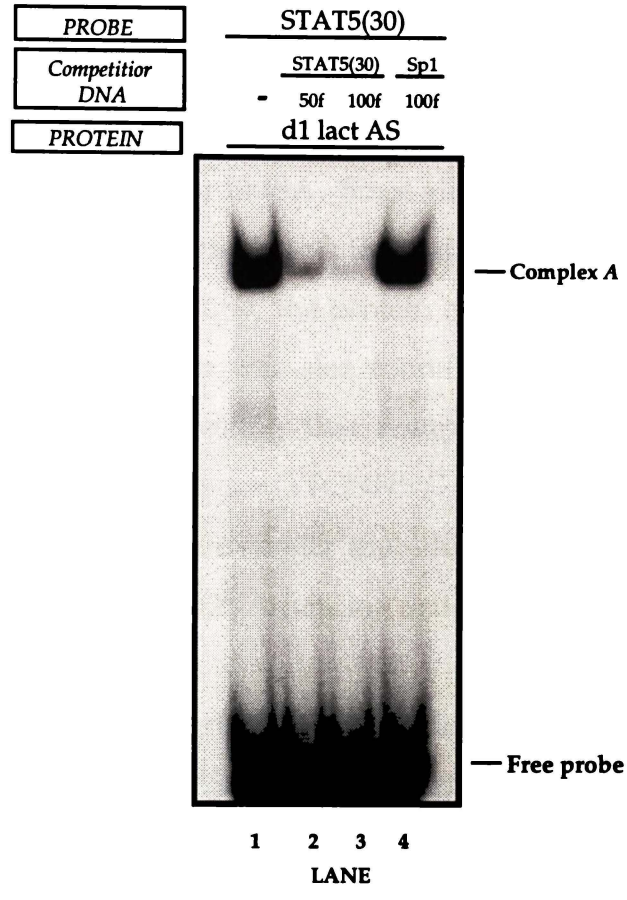


Figure 39 *Excess unlabelled STAT5(30) oligonucleotide competes with complex A for binding to the STAT5(30) probe*

Radioactively labelled STAT5(30) oligonucleotide (lanes 1-4) was incubated with 5μg of ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice. In some cases, either 50- or 100-fold molar excess of unlabelled STAT5(30) oligonucleotide (lanes 2 & 3, respectively), or 100-fold molar excess of unlabelled Sp1 oligonucleotide (lane 4) was added to each EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. 50f, 50-fold; 100f, 100-fold.

Complex *A* has a similar mobility to complex II/NF-κB. To determine whether complex *A* is identical to complex II, EMSA were performed to characterise the binding site for complex *A* within the STAT5(30) probe. The STAT5(30), STAT5(21), STAT5(30-A) and STAT5(30-B) oligonucleotides were used as radioactive probes for EMSA. Since all of the probes span the STAT5 DNA-binding site, STAT5 was found to bind to all of the probes (**Fig. 40**, lanes 3, 6, 9 & 12). Interestingly, the protein complex *A* which is present in ammonium sulphate extracts was also found to bind to all of the probes (lanes 2, 5, 8 & 11), suggesting that complex *A* binds directly to the STAT5/GAS consensus sequence (TTCNNNGAA). Thus, although complex *A* and complex II/NF-κB have similar mobilities, complex *A* is not identical to the CHX-activated NF-κB DNA-binding activity.

To further analyse the protein complex detected in ammonium sulphate extracts, competition EMSA were performed. The unlabelled oligonucleotides used for these experiments were STAT5(30), STAT5(21), STAT5(30-A), STAT5(30-B), m67 SIE, YY1 and NF-κB (**Fig. 33** and **Table 5**). Using the STAT5(30) oligonucleotide as the radioactive probe, excess unlabelled STAT5(30), STAT5(21), STAT5(30-A) and STAT5(30-B) oligonucleotides competed with complex *A* (**Fig. 41**, lanes 3-6). In contrast, excess unlabelled YY1 and NF-κB had no effect on the formation of complex *A* (lanes 8-9), showing that complex *A* does not contain YY1 or NF-κB.

Finally, excess unlabelled m67 SIE competed with complex *A* (lane 7). Since STAT proteins are known to bind to the m67 SIE oligonucleotide (Sadowski *et al.*, 1983; Hill & Treisman, 1995), and STAT1, STAT3 and STAT5 are known to be stimulated in response to prolactin (Bole-Feysot *et al.*, 1998), complex *A* may contain either STAT1, STAT3 or STAT5. Since complex *A* binds to the STAT5 DNA-binding consensus sequence, EMSA were performed using anti-STAT5N antibodies to determine whether complex *A* contained a C-terminally truncated form of STAT5.

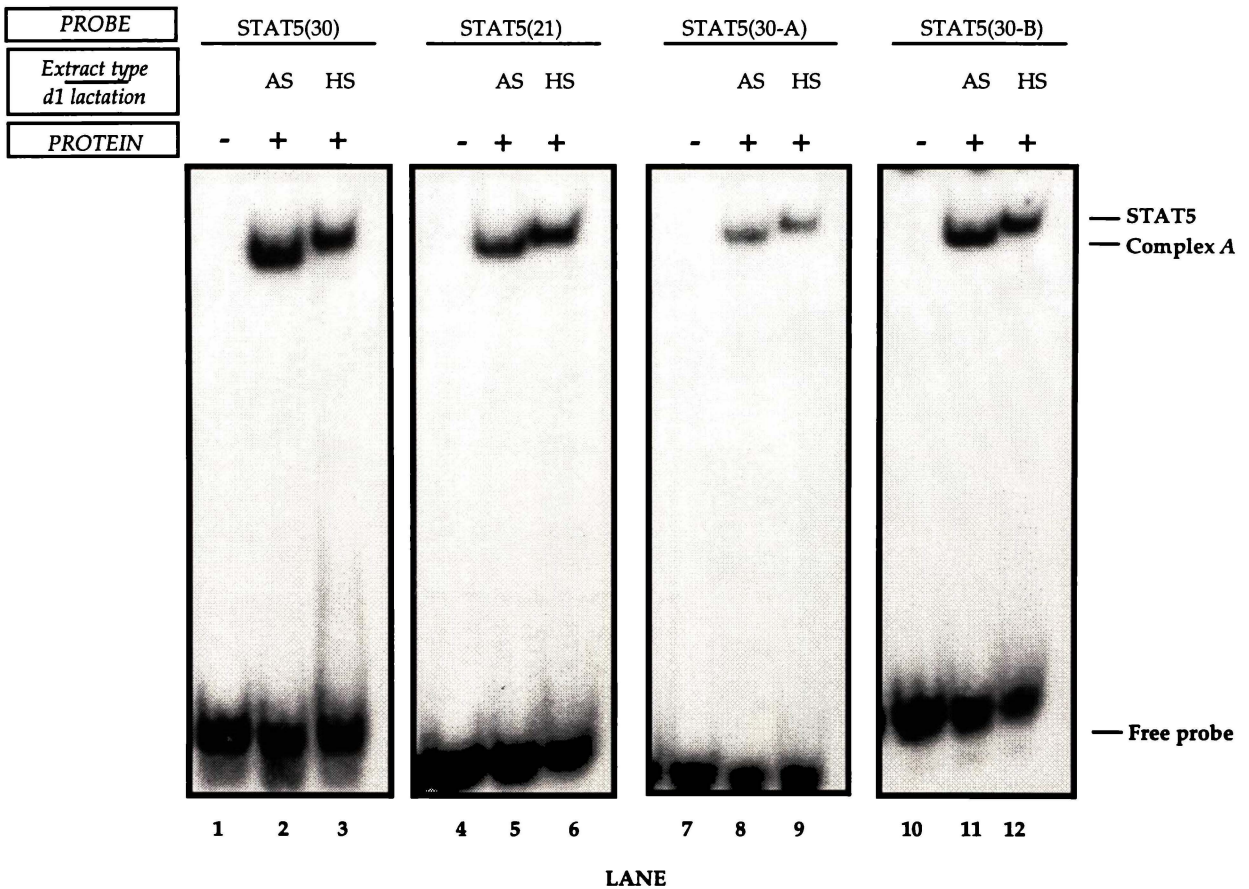


Figure 40 *Complex A binds to the STAT5 DNA-binding consensus sequence*
Radioactively labelled STAT5(30) oligonucleotide (lanes 1-3), STAT5(21) oligonucleotide (lanes 4-6), STAT5(30-A) oligonucleotide (lanes 7-9) or STAT5(30-B) oligonucleotide (lanes 10-12) was incubated with 5 μg of mammary nuclear proteins isolated from d 1 lactating mice by either ammonium sulphate precipitation (lanes 2, 5, 8 & 11), or high salt extraction (3, 6, 9 & 12). Protein-DNA complexes were analysed as described in Fig. 24. Free probe is indicated. AS, ammonium sulphate precipitated nuclear proteins; HS, high salt extracted nuclear proteins.

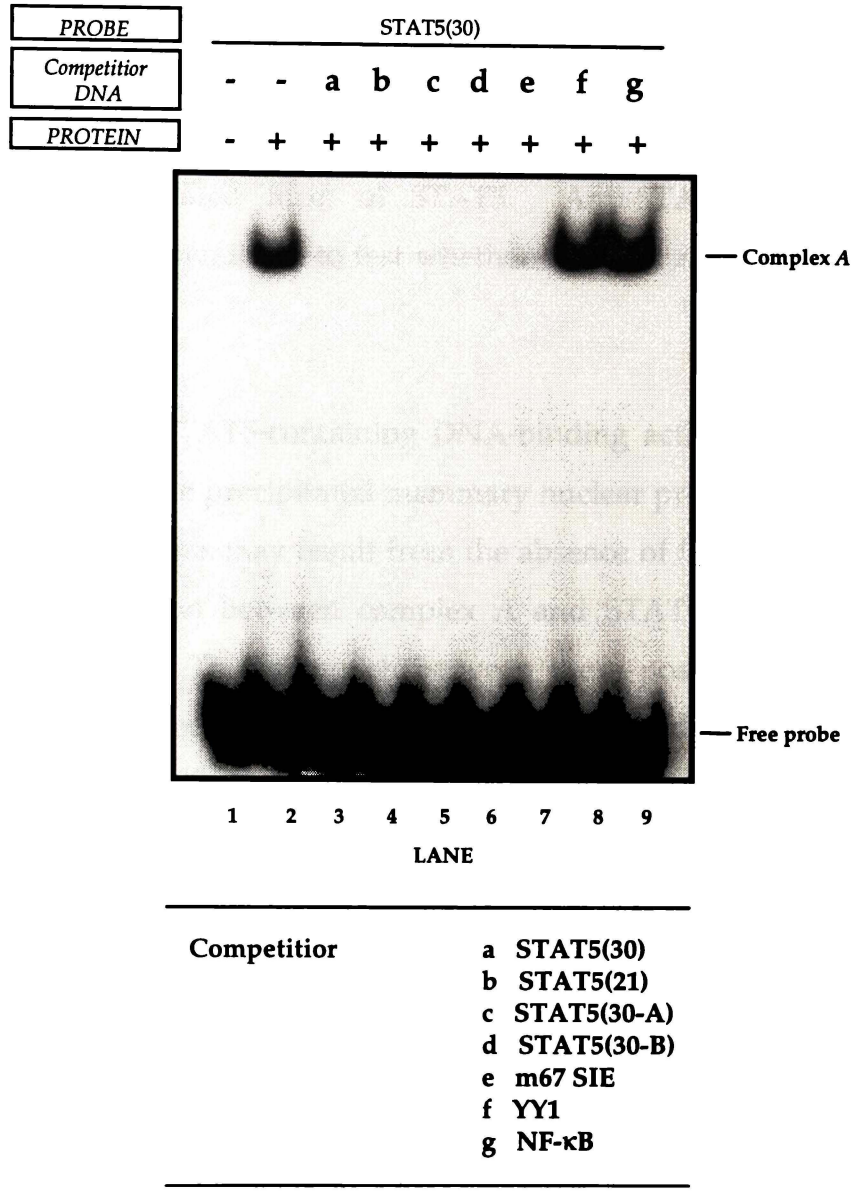


Figure 41 *Excess unlabelled m67 SIE oligonucleotide competes with complex A for binding to the STAT5(30) probe*

Radioactively labelled STAT5(30) oligonucleotide (lanes 1-9) was incubated without protein (lane 1), or with 5μg of ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice (lanes 2-9). Either 100-fold molar excess of unlabelled STAT5(30) (lane 3), STAT5(21) (lane 4), STAT5(30-A) (lane 5), STAT(30-B) (lane 6), m67 SIE (lane 7), YY1 (lane 8) or NF-κB (lane 9) oligonucleotide was added to each EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24.

The binding of complex *A* to the STAT5(30) probe was not affected by the addition of anti-STAT5N antibodies to the EMSA reaction (**Fig. 42**, lane 4). These results suggest that complex *A* does not contain STAT5a, STAT5b or a C-terminally truncated form of STAT5. Anti-STAT1 and anti-STAT3 antibodies were unavailable to test whether complex *A* contained STAT1 or STAT3.

Of note, no STAT5-containing DNA-binding activity was detected in ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice. This may result from the absence of STAT5 in the extracts, or from competition between complex *A* and STAT5 for binding to the STAT5(30) probe. To distinguish between these possibilities, ammonium sulphate precipitated nuclear proteins were subjected to Western analysis using anti-STAT5a antibodies. Equivalent amounts of nuclear proteins were separated by electrophoresis in a 9% (w/v) polyacrylamide-SDS gel and transferred to nitrocellulose membrane. STAT5a was detected in the positive control samples (high salt nuclear extracts isolated from prolactin-treated COMMA-D cells, and mammary tissue derived from d 1 lactating mice, **Fig. 43**, lanes 1, 2, 4 & 5). However, STAT5a was not detected in ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice (lane 7). These results suggest that STAT5a is not present in the ammonium sulphate precipitated nuclear proteins. Thus, in the absence of high levels of STAT5a, complex *A* is able to bind to the STAT5(30) probe. If complex A is present in high salt extracted nuclear proteins isolated from tissue at the same stage of lactation, it does not compete with STAT5 for binding to the STAT5(30) probe.

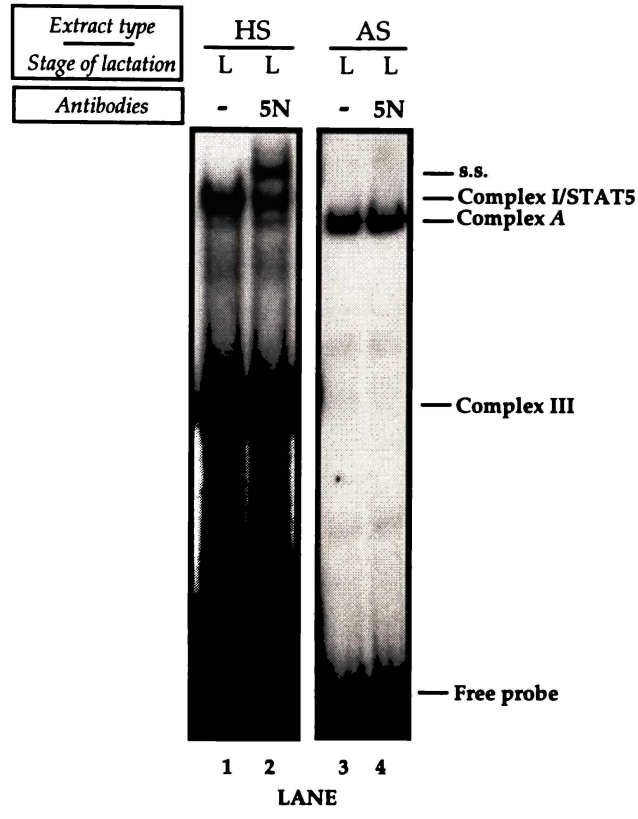


Figure 42 *Complex A does not contain an immunoreactive C-terminally truncated form of STAT5*

Radioactively labelled STAT5(30) oligonucleotide was incubated with 5 μg of high salt extracted mammary nuclear proteins derived from d1 lactating mice (lanes 1-2), or ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice (lanes 3-4). In some cases, 1 μg of anti-STAT5N antibodies (lanes 2 & 4) was added to the EMSA reaction. Protein-DNA complexes were analysed as described in Fig. 24. s.s., 'supershifted' band; 5N, anti-STAT5N antibodies.

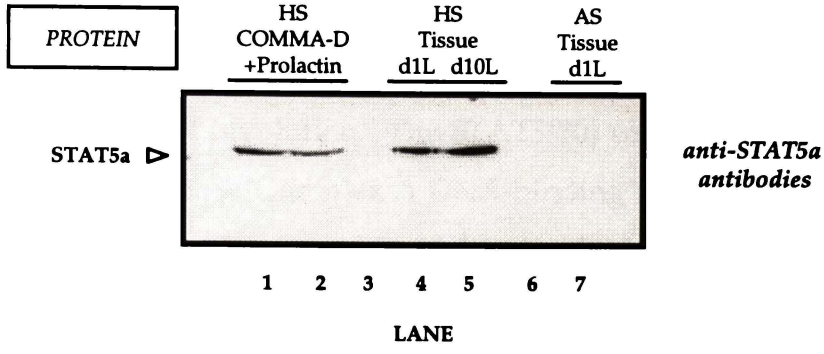


Figure 43 *Immunoreactive STAT5a was not detected in ammonium sulphate precipitated mammary nuclear proteins derived from d1 lactating mice*

Equivalent amounts of nuclear proteins (10 μg) isolated from COMMA-D cells or mammary tissue were subjected to electrophoresis in a 9% (w/v) polyacrylamide gel and transferred to Polyscreen® PVDF membrane for Western analysis. COMMA-D cells were cultured for 2d in the presence of insulin and hydrocortisone, and then for 1h in the presence of 5 μg/mL prolactin. Nuclear proteins were isolated from mammary tissue derived from d 1 or d 10 lactating mice by either high salt extraction (lanes 4 & 5), or ammonium sulphate precipitation (lane 7). The blot was probed with anti-STAT5a antibodies and the signal was detected using enhanced chemiluminescence (ECL) as described in Materials and Methods. d1L, d 1 lactating mice; d10L, d 10 lactating mice; AS, ammonium sulphate precipitated mammary nuclear proteins, HS, high salt extracted mammary nuclear proteins.

To further investigate the role of complex *A* in the mammary gland, complex A activity levels were investigated throughout the lactation cycle. EMSA were performed using nuclear proteins isolated from mammary tissue derived from non-pregnant mice, or mice at d 10 of pregnancy, d 1 and d 10 of lactation and d 5 of involution. The STAT5(30) oligonucleotide was used as the radioactive probe. Complex *A* DNA-binding activity was detected at low levels during pregnancy and at higher levels during lactation (**Fig. 44,** lanes 3-5). However, complex A activity was not detected in nuclear proteins isolated from non-pregnant or involuting mice (lanes 2 & 6). These results suggest that complex *A* may have a role in the lactating gland, however the identity and functional significance of this lactation-associated DNA-binding activity remains to be established.

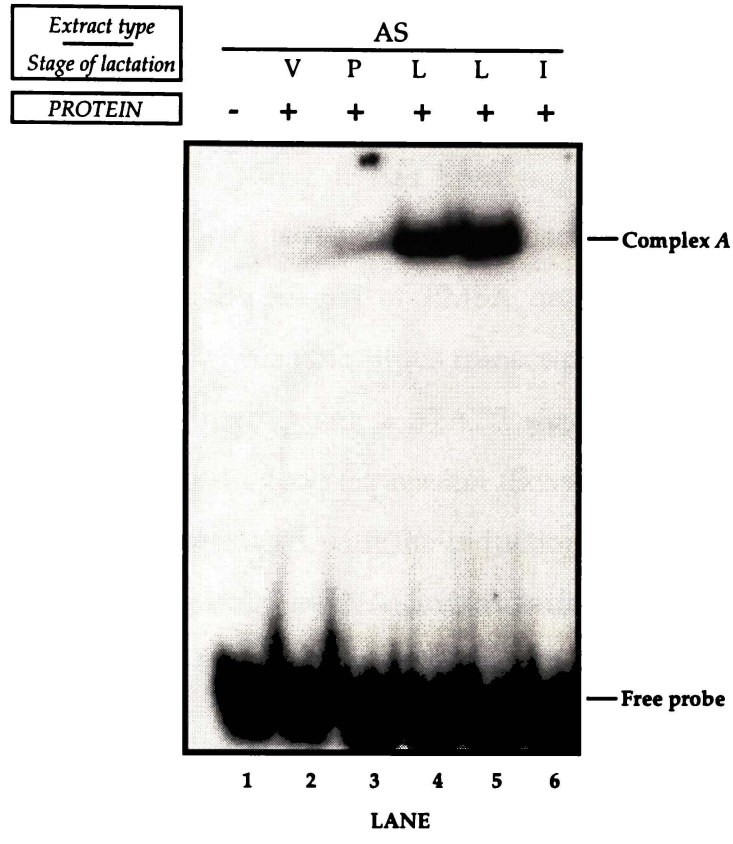


Figure 44 *Complex A activity levels are lactation-specific*

Radioactively labelled STAT5(30) oligonucleotide (lanes 1-6) was incubated with 5μg of ammonium sulphate precipitated mammary nuclear proteins derived from non-pregnant (lane 2), d 10 pregnant (lane 3), d 1 lactating (lane 4), d 10 lactating (lane 5), or d 5 involuting (lane 6) mice. Protein-DNA complexes were analysed as described in Fig. 24. AS, ammonium sulphate precipitated nuclear proteins; V, virgin; P, pregnant, L, lactating; I, involuting.

Discussion

The aim of the experiments presented in this chapter was to define the molecular mechanism whereby CHX inhibits β-casein gene expression in COMMA-D cells. A similar effect has also been observed by others (Yoshimura & Oka, 1990), and it has been suggested that some newly synthesised regulatory factor is required for transcriptional regulation of the β-casein gene. In fact, the results of EMSA analyses showed that CHX stimulates the activation of the ubiquitous transcription factor, NF-κB, which binds to a κB half-site overlapping a STAT5 site previously shown to be crucial for maximal β-casein gene expression (Schmitt-Ney *et al.*, 1991). The activation of NF-κB is correlated with the reduction in β-casein transcription and suppression of β-casein mRNA accumulation in CHX-treated cells, suggesting that this factor may act as an antagonist for STAT5 transactivation, blocking its binding and ability to positively regulate transcription in these cells.

What is the mechanism of CHX action?

STAT5 is known to positively regulate β-casein gene expression via transactivation of the β-casein promoter. Thus, one line of investigation was centred around the possible effect of CHX-treatment on STAT5 protein and activity levels. STAT5 levels were measured using Western analysis, in order to determine whether the pre-existing pool of STAT5 was significantly altered in cells treated with a protein synthesis inhibitor. Since STAT5 is activated via a post-translational mechanism (Gouilleux *et al.*, 1994), it is perhaps not surprising that STAT5 protein levels were not rapidly or significantly altered in response to prolactin- and/or CHX-treatment. In contrast, upon investigation of STAT5 DNA-binding activity, it was shown that CHX inhibited the formation of STAT5 (complex I), and stimulated the activation of a DNA-binding activity of distinct mobility to STAT5, which was able to bind to the STAT5(30)/rat β-casein promoter sequence.

In some cases, it is possible that multiple forms of a protein which carry out one particular function, or closely related functions may bind to a particular nucleotide sequence. Since multiple STAT5 isoforms have been identified, this possibility was explored. In particular, certain STAT5 isoforms have been identified which lack the C-terminal transactivating domain, and consequently act as dominant negative inhibitors of STAT5. It is possible that the binding of a truncated form of STAT5 to the β-casein promoter could result in a dramatic reduction in β-casein mRNA accumulation. However, it was demonstrated that complex II did not contain immunoreactive STAT5a, STAT5b or a C-terminally truncated form of STAT5. In addition, the results of binding studies showed that complex II did not bind directly to the STAT5 site, which is further evidence that this retarded band did not contain STAT5. Thus, the additional DNA-binding activity does not result from genetic redundancy.

Previous studies investigating casein gene expression and the effects of CHX in the cell suggest a number of alternate mechanisms by which CHX could act on β-casein expression. For instance, EGF treatment (Taketani & Oka, 1983) and the overexpression of certain oncogenes (Jehn *et al.*, 1992) are known to inhibit β-casein gene expression in mammary cells in culture. The exact mechanism(s) by which these factors affect β-casein gene expression are currently unknown, but it has been suggested that over-expression of the *mos*, *ras* or *src* (but not *myc*) oncogenes may block the activation of the β-casein promoter via the maintenance of high levels of AP-1 activity (Jehn *et al.*, 1992). Since CHX is able to stimulate the same intracellular signals as EGF, and stimulate the activation of AP-1 DNA-binding activity, the possible implications of these secondary effects of CHX were considered. However, the results of this chapter show that EGF treatment did not appear to exert a significant inhibitory effect on STAT5 DNA-binding activity, or stimulate the formation of complex II/NF-κB. Furthermore, while CHX did stimulate the activation of AP-1 DNA-binding activity, there are no obvious AP-1 binding elements in the STAT5(30)/β-casein promoter sequence. Thus, in the

absence of conclusive evidence supporting the possibility that EGF treatment and/or high levels of AP-1 exert a direct inhibitory effect on the β-casein promoter, the focus of further experiments was to investigate the effect of CHX-treatment on STAT5, and identify the proteins within complex II.

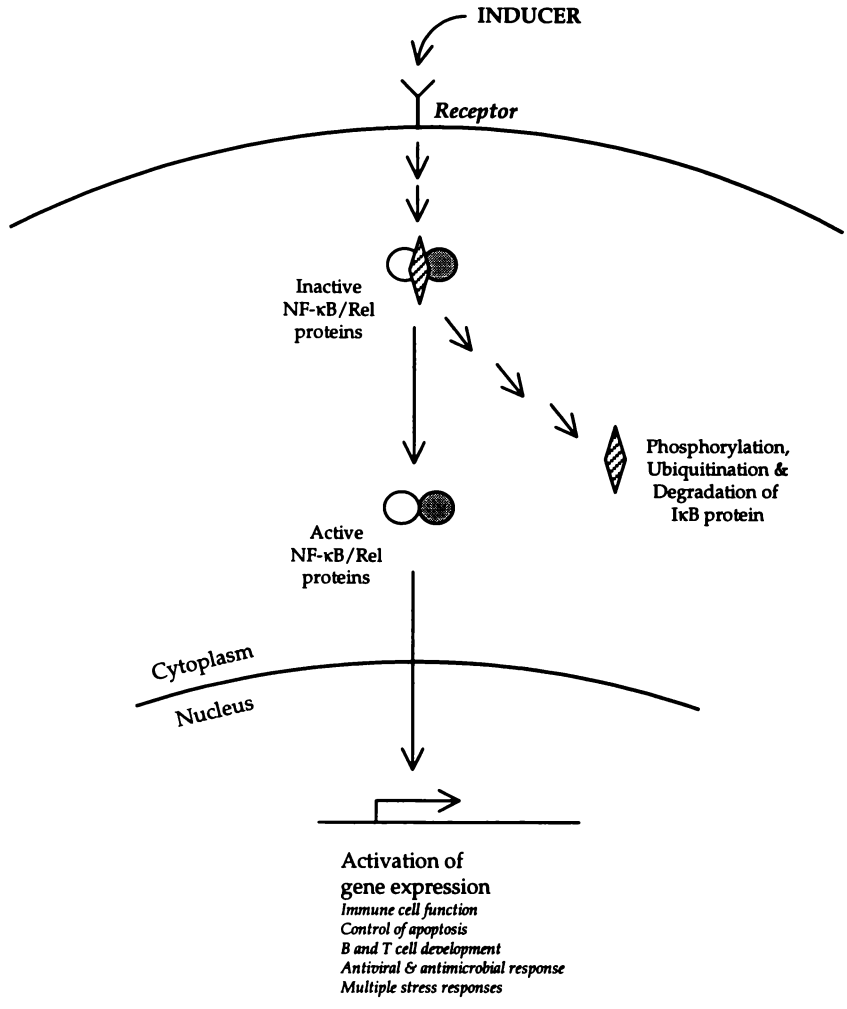
The results of competition EMSA, and the use of antibodies specific to the p50 subunit of NF-κB revealed that complex II contained immunoreactive NF-κB. NF-κB is a heterodimeric, sequence-specific transcription factor and was originally described as a factor which specifically recognised the κ light chain enhancer in B cell lymphocytes (Sen & Baltimore, 1986). NF-κB has since been detected in many cell types (Lenardo & Baltimore, 1989), and belongs to the NF-κB/Rel family of transcriptional regulator proteins. Members of this family include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, cRel and the *Drosophila* proteins Dorsal and Dif. NF-κB is activated by a range of physiological and non-physiological stimuli (for a review see Baldwin, 1996). These include bacterial lipopolysaccharide or inflammatory cytokines such as tumour necrosis factor or interleukin-1, viruses and certain viral gene products, UV irradiation, B or T cell activation, endoplasmic reticulum overloading (Pahl & Baeuerle, 1995; 1997) and protein synthesis inhibitors (Menegazzi *et al.*, 1996; Faggioli *et al.*, 1997). A wide range of genes are regulated by the NF-κB/Rel family, including those involved in immune function, the inflammatory response, cell adhesion, cell growth and apoptosis. In addition, the inappropriate activation of NF-κB has been implicated in a range of diseases (Baeuerle & Baltimore, 1996). An NF-κB site has been identified in the promoter of the β-lactoglobulin gene (Burdon *et al.*, 1994b), however NF-κB recognition sequences do not appear to be widespread within the promoters of the other milk protein genes (Malewski, 1998).

Under normal conditions, NF-κB exists in the cytoplasm in an inactive complex with the inhibitor protein IκB (Baeuerle & Baltimore, 1988).

Interactions between NF-κB and IκB are thought to be the primary level of control for NF-κB activation (for a review see Baldwin, 1996). Five IkB proteins are involved in controlling the activation of NF-κB dimers, IκBα, -β, -γ, -δ and -ε. Extracellular signals trigger the phosphorylation and rapid degradation of IκB (Stancovski & Baltimore, 1997), resulting in the release of NF-κB. The released NF-κB subunits form homo- and hetero-dimers that translocate to the nucleus and bind to κB elements (consensus sequence GGGAATTCCC), transactivating the downstream genes (see **Fig. 45**). It has recently been reported that activated NF-κB factors are also able to associate with a κB half site, GGGAAT (Murphy *et al.*, 1995), similar to that found in the rat β-casein promoter/STAT5(30) nucleotide sequence, GGAATT.

How does CHX stimulate the activation of NF-κB?

Due to a constitutively low level activation of NF-κB in cells (Rice & Ernst, 1993), *de novo* synthesis of IκB is required to form the inactive, cytoplasmic complex. Therefore, it is possible that CHX treatment results in the rapid loss of IκB in COMMA-D cells, disrupting the potential negative feedback loop of *de novo* IκB synthesis which is required to down-regulate NF-κB. This could be confirmed by measuring IκB levels using Western analysis. Anti-IκB antibodies were not available to test this hypothesis here, however Newton *et al.* (1996) have shown that CHX stimulates the activation of NF-κB via this mechanism in the human pulmonary epithelial cell line, A549. Similarly, CHX is known to activate the transcription of some other genes which are kept inactive in quiescent cells by the continuous synthesis of labile repressors, or via autorepression (Wall *et al.*, 1986; Greenburg *et al.*, 1986). Thus, this is likely to be the mechanism by which CHX stimulates NF-κB activation in COMMA-D cells.



adapted from Baeuerle & Baltimore, (1996) and Baldwin, (1996)

Figure 45 *Generalised NF-κB activation scheme*

NF-κB dimers exist as an inactive complex with the inhibitor protein, IκB in the cytoplasm of the cell. Following exposure of a cell to an inducer, IκB becomes phosphorylated, ubiquitinated and degraded. Activated NF-κB dimers then translocate to the nucleus to activate a variety of genes.

Does NF-κB-STAT5 antagonism occur and is this functionally significant?

It was not conclusively shown in this study that activation of NF-κB, and competition between STAT5 and NF-κB for binding to the β-casein promoter results in the reduction of β-casein gene expression in CHX-treated cells. However, the correlation between the reduction in β-casein gene expression, NF-κB activation and the apparent mutually exclusive binding of STAT5 and NF-κB, suggests that NF-κB activation is one potential mechanism to explain the findings presented in Chapter 4. This possibility could be tested *in vivo*, by performing site-directed mutagenesis on a β-casein promoter-reporter construct. According to the STAT5-NF-κB antagonism hypothesis, point mutations which mutate the κB site but retain the STAT5 site should abolish the effect of CHX on β-casein gene expression in cells transfected with these constructs. Until this hypothesis can be proven, it is important to consider that CHX-activated NF-κB could be an example of an *in vitro* artefact that would not bind to the STAT5(30)/rat β-casein promoter sequence, or have a functional role *in vivo*.

In cases where DNA-binding motifs are overlapping and there is insufficient space for mutual binding, the binding preference is probably related to the relative concentrations of the proteins within the nucleus. If NF-κB activation does mediate the effect of CHX on β-casein transcription, certain physiological signals may stimulate the activation of NF-κB to levels high enough to compete with STAT5 for binding to target genes, and impact on mammary function. As discussed earlier, NF-κB is known to be activated in response to inflammatory cytokines and bacterial toxins, which highlights the importance of this factor during the inflammatory process. This may be of particular relevance as inflammation is associated with mammary infection and injury (mastitis). Mastitis can affect the gland to varying degrees, but is usually characterised by gross swelling, heat, redness, pain and in particular, impaired function. Thus, it is possible that NF-κB-STAT5 antagonism (in addition to other influences), could contribute to the down-

regulation of milk protein synthesis/production and changes in milk composition in the mastitic gland. Therefore, it would be useful to analyse complex I and complex II formation during mastitis and involution.

NF-κB has also been shown to be activated in response to intracellular stresses such as ER overloading of secretory proteins. It is thought that NF-κB participates in an ER-nuclear signal transduction pathway which mediates changes in the pattern of gene expression. This signalling pathway is distinct to the unfolded-protein-response pathway which activates the expression of the *grp* genes (Kozutsumi *et al.*, 1988). Higher eukaryotes are capable of expanding their ER content when the amount of protein requiring ER processing exceeds the capacity of this organelle (Shands *et al.*, 1973). It has been suggested that NF-κB could trigger ER biosynthesis to relieve ER congestion (Pahl & Baeuerle, 1995; 1997). In addition, NF-κB-STAT5 antagonism may also provide a potential mechanism to down-regulate the synthesis of secretory milk proteins under these conditions.

Thus, in certain cases NF-κB-STAT5 antagonism may be important for the local regulation of milk protein gene expression in the mammary gland. This possibility is supported by emerging evidence which highlights the importance of interactions between NF-κB and STAT factors to regulate gene expression in several other tissues and cell types. For example, Zhang & Fuller (1997) have shown that IL-1 activated NF-κB and IL-6 activated STAT3 derived from rat primary hepatocytes, compete for binding to overlapping sites in a sequence derived from the α2-macroglobulin gene promoter. This provides a potential mechanism for the regulation of gene expression during acute inflammation. In addition, Bennett *et al.*, (1997) demonstrated that IL-4 suppression of tumour necrosis factor α-stimulated E-selectin gene transcription is mediated by STAT5 antagonism of NF-κB in human umbilical vein endothelial cells. This is another example of an antagonistic relationship between NF-κB and STAT5, and interestingly also demonstrates

that a STAT factor can act as a transcriptional repressor rather than an activator. In contrast, Messner *et al.*, (1997) showed that co-operation between STAT6 and NF-κB is important for IL-4-dependent activation of the IgE germline gene in a human lymphoma cell line. In a broader sense, these results highlight the importance of complex antagonistic or synergistic protein-protein interactions, in addition to simple DNA-protein interactions, for the regulation of gene expression.

Consensus DNA motifs specifically recognised by STAT factors have been identified in the promoters of a wide array of target genes. As discussed in Chapter 2, the motif, termed GAS (γ-interferon-associated sequence) consists of a palindromic sequence TTCNNNGAA. It is thought that the specificity of the interaction between a particular STAT factor and a GAS motif may, in part, depend on the three centre nucleotides. Given the fact that all STAT factors bind to similar DNA binding motifs which may inherently contain a κB half-site, and that STAT factors are activated by a variety of cytokines and growth factors, the findings presented in this thesis may offer a possible control mechanism that can be extended to other cytokine regulated genes.

A preliminary search for NF-κB-STAT5 antagonism in the mammary gland *in vivo* revealed a lactation-specific DNA-binding activity (complex *A*), of similar mobility to complex II/NF-κB, which binds to the STAT5(30)/β-casein promoter sequence. However, it was shown that this DNA-binding activity did not contain NF-κB. Complex *A* was not definitively identified, but the results of competition EMSA suggest that complex *A* may contain STAT1 or STAT3. Of note, complex *A* was detected in ammonium sulphate precipitated mammary nuclear proteins derived from d 1 lactating mice, but no STAT5 activity was detected in these same extracts. Since STAT5 could not be detected in these proteins using Western analysis, and complex *A* activity was not detected in high salt extracted nuclear proteins isolated from the same tissue, it is likely that complex *A* is only able to bind to the STAT5

consensus sequence in the absence of high levels of STAT5. It is interesting to note that three STAT factors, STAT1, STAT3 and mainly STAT5 have been identified as transducer molecules of the prolactin receptor (Bole-Feysot *et al.*, 1998). Thus, it is possible that complex *A* mediates the effects of prolactin in the lactating mammary gland by stimulating the transcription of certain lactation-specific target genes.

These results emphasise that the quality of nuclear protein extracts can be a limiting factor when interpreting the results of EMSA analyses. Ammonium sulphate precipitated nuclear proteins have been used previously to investigate the role of MGF (now known as STAT5) in the mammary gland (Schmitt-Ney *et al.*, 1991; 1992). However, in these studies the binding factor was not identified as MGF-STAT5 by supershifting with antibodies specific for this protein. This is most important since all of the STAT factors (excluding STAT2) have very similar or identical binding sites, and the current findings draw attention to the possibility that this DNA-binding activity may have been, in fact, another STAT factor.

In summary, from the results of preliminary investigations *in vivo*, there is no evidence yet to support that competition between NF-κB and STAT5 is functionally significant in the mouse mammary gland. Nevertheless, this possibility is an attractive one since not only would competition between CHX-activated NF-κB and prolactin-activated STAT5 provide an explanation for the substantial reduction in β-casein mRNA accumulation, but these results may have uncovered a novel control mechanism.

A wider search may confirm a functional role for overlapping STAT5 and κB half-sites in the promoters of the milk protein genes, and a possible role for NF-κB in the modulation of milk protein gene expression. Although prolactin treatment did not stimulate the activation of NF-κB in COMMA-D cells, experiments could be designed to investigate the hormonal

responsiveness of NF-κB *in vivo*. This could be determined by performing EMSA to analyse NF-κB DNA-binding activity over the lactation cycle.

Is protein synthesis required for β-casein gene expression?

Clearly, the importance of rapid responses to prolactin via pre-existing proteins has been established. However, to achieve specific responses using common sets of signalling molecules such as the JAKs and STATs, a number of other factors must work in concert with STAT5 to regulate transcription at the promoter. It is possible that the synthesis of some of these factors is required to achieve a maximal response to hormonal stimulation. In a broader sense, maximal β-casein gene expression results from the co-ordination of several controlling mechanisms including gene transcription, mRNA stability, translation, secretion and protein degradation, in addition to higher order levels of regulation, such as changes in chromatin-structure. Protein synthesis is undoubtedly required at many steps in this overall process. In fact, there is evidence to support that steroid hormone-dependent induction of β-casein gene expression is prevented by CHX, and that a labile protein factor is required to stabilise β-casein transcripts in mammary cells treated with glucocorticoids (Poyet *et al.*, 1989).

The current findings offer a possible explanation as to the requirement for protein synthesis during lactogenic signalling in mammary epithelial cells. However, if NF-κB antagonism of STAT5 activity is responsible for the CHX-dependent decrease in β-casein transcription, then the requirement for ongoing protein synthesis to achieve maximal β-casein gene expression remains an open question.

Chapter Six - Concluding discussion

The project described in this thesis explored the *de novo* synthesis of proteins in response to prolactin in mouse mammary epithelial cells, with the aim of identifying factors which are involved in mediating the lactogenic effects of prolactin.

Two prolactin-responsive and lactation-associated proteins were successfully identified, namely, glucose regulated protein (Grp78) and protein disulphide isomerase (PDI). These proteins are reticuloplasmins and are known to participate in several metabolic processes within the endoplasmic reticulum of many cell types, and are likely to have similar, co-ordinated roles processing milk proteins for secretion in mammary epithelial cells. The current investigations demonstrate that they are not rapidly induced in response to hormonal stimulation, and are thus unlikely to act as signalling molecules for prolactin. Nevertheless, since grp78 and PDI are potential regulators of lactation at the cellular level, it may be possible to manipulate the levels or functions of these proteins in the mammary gland, in order to alter the properties or composition of milk in a beneficial way.

It is well established that rapid responses to extracellular stimuli are often mediated by pre-existing factors (such as STAT5), which are activated by post-translational modifications. However, since the *de novo* synthesis of transcription factors is also a common control strategy (especially during differentiation processes), it might be expected that the ongoing synthesis of a number of proteins is required to mediate hormonal signals in the mammary gland. Indeed, it has been demonstrated that protein synthesis is required to mediate steroid-hormone responses in mammary cells in culture (Poyet *et al.*, 1989). In the present thesis, the results of investigations designed to address

this question in prolactin-stimulated COMMA-D cells suggested that ongoing protein synthesis is required for maximal β-casein gene expression at the level of transcription. This is in agreement with the results of other researchers who have addressed similar questions (Yoshimura & Oka, 1990; Goodman & Rosen, 1990). However, no such newly synthesised proteins were identified as part of the studies of Yoshimura & Oka, (1990). Therefore, the results presented in this thesis go further towards defining the requirement for protein synthesis, by demonstrating that the effect of protein synthesis inhibition on prolactin-induced β-casein gene expression was independent of the lactogenic hormones, insulin and hydrocortisone, and the extracellular matrix.

During the course of experiments aimed at defining the molecular mechanism of CHX action in mammary cells, it was demonstrated that CHX stimulates the activation of NF-κB. This has not been shown in mouse mammary epithelial cells. It is likely that CHX stimulates the activation of NF-κB by preventing the synthesis of the NF-κB inhibitor protein IκB, thereby allowing NF-κB to bind to a putative κB half-site in the β-casein promoter, which overlaps a crucial STAT5 site (located between positions -104 to -75). It is possible that the binding of activated NF-κB to the β-casein promoter sterically hinders STAT5 binding, and transactivation of β-casein gene transcription. A final confirmation of this interpretation will require analysis of whether or not NF-κB can effectively compete with STAT5 for binding to overlapping consensus sequences, and whether NF-κB binding at this site affects promoter function in transactivation assays. Furthermore, although NF-κB was activated in response to a non-physiological stimulus (CHX), a more extensive investigation may reveal novel situations in which NF-κB is activated to high enough levels to compete with STAT5, and modulate milk protein gene expression *in vivo*. Nevertheless, if NF-κB activation is responsible for the decrease in β-casein gene expression in CHX-treated COMMA-D cells, the requirement for ongoing protein synthesis during prolactin signalling remains an open question.

It may be valuable to use the proteome analysis approach to investigate more rapid prolactin-induced changes in protein synthesis in order to identify novel proteins. Similarly, alternative methods could be employed to analyse differences in gene expression between prolactin-treated and control cells, or lactating and non-lactating cells. Representational difference analysis or differential display methodologies may be suitable, but care would be needed in selecting two appropriate populations of cells for comparison, in order to maximise the likelihood of detecting genes encoding signalling molecules and transcription factors. Cell lines offer an advantage here, since the cells are more homogeneous than comparing cells from different animals at different lactation states. Alternatively, a technique known as serial analysis of gene expression (SAGE) has been described (Velculescu *et al.*, 1995), which allows the detailed and rapid analysis of thousands of transcripts expressed in cells in different developmental or disease states. SAGE is based on the concatenation of short nucleotide sequence tags which allows efficient, serial analysis of multiple tags within a single cloned DNA construct. Therefore, this approach may provide more insight into the extent of gene expression differences underlying changes in lactation state and reveal genes which are involved in regulating milk protein gene expression. Finally, microarray technology using immobilised cDNA to simultaneously monitor the expression of many (differentially expressed) genes, is an extremely powerful tool for gene discovery (Ramsay, 1998; Schena *et al.*, 1998; Chen *et al.*, 1998). More generally, this, or other approaches could be directed towards analysing the differences in gene expression between cells in other biological/lactation states.

Despite the considerable range of general and specific effects of protein synthesis inhibitors such as cycloheximide in the cell, this drug has been routinely used to test the requirement for ongoing protein synthesis in a range of other tissues and cell types, and in particular, to delineate the pleiotropic effects of hormones. Indeed, prolactin exerts a wide range of effects in the cell, and so cycloheximide is a useful tool to dissect the primary (rapid) effects of prolactin from the secondary responses, which require ongoing protein

synthesis. This approach could be used to investigate the effects of other hormones on milk protein gene expression.

In summary, the results of the experiments described in this thesis contribute to the growing understanding of how lactation is regulated at the cellular and molecular level. More specifically, two prolactin-induced proteins have been identified as glucose regulated protein 78 and protein disulphide isomerase, endoplasmic reticulum proteins that probably function in the processing of milk proteins for secretion. The dependence on ongoing protein synthesis for maximal β-casein gene expression has been characterised, and detailed investigation has revealed the probable molecular mechanism driving the requirement for ongoing protein synthesis; active synthesis of the NF-κB inhibitor protein, IκB, which blocks interaction of active NF-κB to a binding site in the β-casein promoter thereby inhibiting STAT5 binding at an overlapping site. It is increasingly becoming recognised that the interplay between transcription factors is important for the regulation of gene expression at proximal promoters. Thus, NF-κB-STAT interactions may be an example of another mechanism used to regulate gene expression in other tissues and cell types, which may be extended to the mammary gland. The results presented in this thesis provide the platform to investigate the role of NF-κB during lactation in more detail.

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Appendix 1

Antibodies

Appendix 1 *Antibodies*

Primary Antibodies	*Source*
STAT5a (sc-1081) *TransCruz*™ Gel Supershift reagent, specific for STAT5a, non cross-reactive with STAT5b (epitope corresponding to amino acids 774-793 at the carboxy terminus of STAT5a of mouse origin), rabbit polyclonal IgG	Santa Cruz Biotechnology, Inc., CA, USA
STAT5b (sc-835), specific for STAT5a and STAT5b, (epitope corresponding to amino acids 711-727 at the carboxy terminus of STAT5b of mouse origin), rabbit polyclonal IgG	Santa Cruz Biotechnology, Inc., CA, USA
STAT5b ***Note that this antibody is denoted STAT5N in the text.*** (sc-836) *TransCruz*™ Gel Supershift reagent, specific for STAT5a and STAT5b,(epitope corresponding to amino acids 5-24 at the amino terminus of STAT5b of mouse origin), rabbit polyclonal IgG	Santa Cruz Biotechnology, Inc., CA, USA
NF-κB p50 (NLS) (sc-114) *TransCruz*™ Gel Supershift reagent, specific for NF-κB, (epitope corresponding to amino acids 350-363 within the NLS region of NF-κB p50 of human origin, identical to the corresponding mouse sequence), rabbit polyclonal IgG	Santa Cruz Biotechnology, Inc., CA, USA
Secondary Antibodies	
Anti-Rabbit IgG Peroxidase conjugate. Antibody developed in goat. Affinity isolated antigen specific antibody	Sigma Chemical Co., CA, USA
Anti-Goat IgG Peroxidase conjugate. Antibody developed in rabbit. Affinity isolated antigen specific antibody	Sigma Chemical Co., CA, USA

Appendix 2

Publication of Beaton *et al.*, 1997