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REGULATION OF TRANSCRIPTION FACTOR STAT5 IN THE
RAT AND BOVINE MAMMARY GLAND

by

JINZENG YANG ©

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

Doctor of Philosophy

In

Animal Science

Department of Agricultural, Food and Nutritional Science

Edmonton Alberta
Spring 2000

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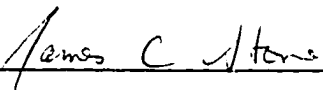
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ABSTRACT

Stat5 belongs to the molecular family of signal transducers and activators of transcription (Stat). It is involved in the signaling pathways of multiple hormones and growth factors including prolactin (PRL) and growth hormone (GH) in transfected cells. One form of Stat5 deficiency results in failed lactation in mice, suggesting its critical role for normal mammary function. The specific roles of Stat5 and its *in vivo* regulation are not well understood in bovine mammary gland. This thesis was designed to investigate the hypothesis that Stat5 may serve as a common point in the signal pathways of various physiological stimuli in the mammary cells. Assays for Stat5 DNA binding activity and protein were validated in mammary explant culture. Both rat and bovine explant culture showed a rapid stimulation of Stat5 DNA binding activity by PRL, GH and Insulin-like growth factor-I (IGF-I), at the high concentrations typically used in explant cultures as well as at the levels within physiologic ranges. Growth hormone stimulated Stat5 activity at a lower concentration in bovine than in rats, but in both species the presence of GH increased the response of Stat5 activity to PRL, showing additive effects of PRL and GH action on Stat5 activation.

The bovine mammary gland was used to investigate physiological regulation of Stat5 activity *in vivo*. Mammary Stat5 DNA binding activity was absent in non-lactating and non-pregnant cows, and present in late pregnancy and throughout lactation. The activity of Stat5 varied among cows in similar stages of lactation. To investigate the physiological source of the variations, Stat5 was detected in hormone-

treated cows and mammary quarters milked by different frequencies. Infusion of GH and GH-releasing factor for two months significantly raised levels of milk protein production and depressed mammary Stat5 activity without influencing Stat5 protein abundance. Mammary Stat5 was also influenced by milking frequency; once daily milking significantly reduced Stat5 activity and protein abundance compared with twice daily milking (P<0.01). Analysis of mammary Stat5 activity in relation to milk protein production indicates that it is correlated (r=0.5, P<0.05) with average milk protein concentration, but not related to milk protein yield.

These results obtained from *in vitro* explant culture and *in vivo* Stat5 measurements demonstrate that Stat5 responds to a number of physiological stimuli. Transcription factor Stat5 may represent part of a common route by which different extracellular signals converge and are transduced intracellularly to coordinately regulate cell function in the mammary gland. However, the levels of Stat5 activity in the mammary gland is not consistently coupled with milk protein synthesis, indicating the roles of other factors other than Stat5 in controlling milk protein production.

ACKNOWLEDGEMENTS

First, and always to my supervisors-Dr. Vickie E. Baracos and Dr. John J. Kennelly, whose academic wisdom and skills have amazed and taught me so much. Without your superb guidance, encouragement and loving care throughout my Ph.D. program, there would not have been a thesis at all. Thank you!

To Drs. James Stone (supervisory committee), Colin Wilde (external examiner) and Stephen Moore, sincere thanks for reviewing the thesis and serving in the Ph.D. final examination committee. To Drs. Allen Tucker, Robert Christopherson and Stephen Rice, thank you for serving on my Ph.D. candidacy examination committee. To Dr. David Begg, thank you for helping me to identify mammary cell nucleus.

The quality of data in this thesis was greatly enhanced by research interest and the technical assistance from the Department. Special thanks to Drs. George Foxcroft, Walter Dixon, Reza Khorazani, David Glimm, Fengqi Zhao, Annette Suominen, Denis Balcerzak, Olasunkanmi Adegoke and Fan Dong, the technical staff Renate Meuser, Jody Aldrich, Donna Talylor, Naomi Beswick, Yuwei Zhao, Brenda Tchir, the graduate students Emma Clowes, Yan Zhang, John Bell, Prasanch Chelikani, Lorraine Doepel and Rick Corbett, Jeff Kaufmann, and the staff of the Dairy Research and Technology Centre. To all Chinese graduate fellows in the Department, thanks for your encouragement and friendship.

My family has been exceptionally supportive during my Ph.D. program. My wife, Baoping Zhao, has made tremendous contributions by her constant love and

technical support. I am also especially thankful to my parents for their encouragement

in my education.

Last, definitely not least, I am grateful for the financial support from the

University of Alberta Ph.D. scholarship and Alberta Agricultural Research Institute.

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CONTENTS

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LIST OF ABBREVIATIONS

bp =	base pair
BSA =	bovine serum albumin
cpm =	counts per minute
CAT=	chloramphenicol acetyltransferase
DNA =	deoxyribonucleic acid
DTT =	dithiothreitol
EDTA =	ethylenediaminetetraacetic acid
EGF =	epidermal growth factor
EMSA=	electrophoresis mobility shift assay
GH =	growth hormone
GHRH=	growth hormone releasing factor
h =	hour
Hepes=	N-[2-Hydroxyethy]piperazine-N'-[2-ethanesulfonic acid]
IGF-I =	insulin-like growth factor-I
Jak2 =	Janus kinase 2
Kb =	kilo bases
mRNA =	messenger ribonucleic acid
PRL=	prolactin
PMSF =	phenylmethysulfonyl fluoride
SEM =	standard error of mean
Stat5 =	signal transducer and activator of transcription 5
TF=	transcription factor
PTP=	protein tyrosine phosphatase

CHAPTER 1. LITERATURE REVIEW

1.1. INTRODUCTION

Milk from dairy cows is an important source of nutrients for humans. In the last several decades, milk yield has substantially increased with improved breeding and nutritional technologies. Average milk yield presently is over 8000 kg per cow per lactation in Alberta. In recent years, health professionals have recommended reducing fat intake so that low fat milk and dairy products with an increased ratio of protein to fat have become popular. This market trend increases the emphasis on high protein content in the milk. Therefore, more research attention is being focused on understanding the regulation of milk protein synthesis.

Milk is synthesized and secreted in the mammary gland. Hormonal and nutrient supplies to the mammary gland regulate milk component synthesis such as milk protein. Increasing nutrient supplies such as amino acids provides an easy way to stimulate milk protein production, but it does not always yield positive results. Current literature indicates that milk protein production shows substantial variation and poor reproducibility in response to nutritional manipulation. These inconsistencies are not surprising given the limited understanding of the underlying biological mechanisms regulating milk protein synthesis in the mammary gland. Therefore the focus of this thesis is on understanding the fundamental biological mechanisms of milk protein synthesis.

Hormonal control of the mammary gland has been widely studied in rodents and tissue culture. Progress in this area of research includes the identification of several hormones and growth factors that regulate lactogenesis and lactation.

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Recently, it has been discovered that a key regulatory factor of gene expression, **signal transducer and activator of transcription 5 (Stat5)**, is activated by multiple hormones and growth factors in transfected mammalian cells (Groner et al., 1994). The DNA binding sequence for Stat5 is conserved in the promoter regions of several major milk protein genes. Lactogenic hormones and some growth factors bind their respective receptors and then activate a protein kinase, thereby inducing Stat5 activation. The current understanding of Stat5 and its function substantiate the hypothesis that it may be a potential candidate involved at the molecular level in the regulation of the mammary function. The physiological regulation of Stat5 such as its response to hormone infusion and *in vivo* changes over the stage of lactation in bovine mammary gland remains unknown. Hence the focus of this thesis is to investigate the Stat5 response to lactogenic hormones and its physiological regulation within the mammary gland.

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1.2. THE MAMMARY GLAND

Milk is synthesized and secreted by mammary glands of lactating animals. Mammary glands achieve their maximal developmental potential following the onset of pregnancy. The lobulo-alveolar structures form around the time of parturition, and then enter a differentiated stage for milk synthesis during lactation. Lactation is initiated and maintained primarily by hormones and growth factors. The synthesis of milk protein is one unique characteristic of the mammary gland. Milk protein gene expression has been established as an experimental model for hormonal regulation of the mammary gland to study their coordinate controlling mechanism. Gene expression is the first essential step in conversion of genetic information in the DNA into protein. Hence, an understanding of milk protein gene expression is an important step toward agricultural control of the mammary gland.

1.2.1. Milk Protein

Milk protein consists of two general classes: casein and whey. Caseins include α_{s1}-casein, β-casein, α_{s2}-casein, κ-casein, and γ-casein, whereas β-lactoglobulin and α-lactoalbumin are the primary whey proteins. γ-Casein is the C-terminal fragment of β-casein released by enzymatic action of plasmin in bovine milk. Among them, α_{s1}-casein and β-casein are the major milk protein components, each representing 30% of the total protein content. Casein proteins are phosphorylated and present in the form of micelles in the milk. Micelles are aggregated by α_{s1}-casein, β-casein and α_{s2}-casein, and coated with κ-casein. Caseins have low solubility at pH 4.5-5.0, and therefore are defined as the proteins that precipitate from milk at pH 4.6 and 40°C. When casein is

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precipitated from milk by acid; the remaining soluble proteins are named whey proteins. Whey also includes serum albumin, iron binding protein-transferrin, lactoferrin, immunoglobulins (colostrum), fat globule membrane proteins, and enzymes, in addition to β-lactoglobulin and α-lactoalbumin.

1.2.2. Endocrine Control of the Mammary gland

Earlier this century Halban (1900) demonstrated that oophorectomy caused mammary regression and transplantation of ovaries prevented the abnormal atrophy of the mammary gland. Later, pituitary extracts were found to induce milk secretion, and prolactin (PRL) was discovered in pituitary gland (Riddle et al., 1933). Since then multiple lactogenic hormones and growth factors have been known to involve in the regulation of lactation.

Transcriptional regulation of β-casein gene has been established as an experimental model of lactogenic hormone action in mammary explant culture (Ball et al., 1988; Doppler et al., 1989; Goodman and Rosen 1990 and Schmidhauser et al., 1990). Prolactin, insulin, and glucocorticoid are three essential hormones for β-casein RNA synthesis in mouse mammary explants (Juergens et al., 1965). In mammary organ culture from pregnant rats, prolactin rapidly (within 1 h) induced the accumulation of β-casein mRNA and the response increased for up to 48 hr. The steroid hormones, hydrocortisone and progesterone, were able to upregulate the prolactin-induced casein mRNA level, and the presence of hydrocortisone was required for maximal levels of β-casein mRNA accumulation (Matusik and Rosen 1978). Further study indicated that PRL and glucocorticoid enhanced both β-casein gene transcription and the stability of the mRNA (Guyette et al., 1979). Glucocorticoid

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and PRL were absolutely required for expression of the β-casein gene in mammary explants. Medium containing insulin and PRL or insulin and cortisol failed to produce β-casein mRNA above the basal level, but when cortisol was added to the medium containing PRL and insulin, a 20-fold increase in β-casein mRNA over the basal level was observed (Ganguly et al. 1980). In 1990, Yoshimura and Oka found that an increase in the β-casein transcript was measurable as early as 30 min after induction with the hormones and the level of mRNA at 24 h of incubation increased more than 100 fold over the initial value. They found that protein synthesis inhibitor-cycloheximide prevented the increase of β-casein gene transcription and speculated newly synthesized proteins might be involved in the activation of lactogenic hormone action on milk protein gene expression.

In mammary cell culture these three hormones were also able to induce β-casein synthesis. In the HC11 mouse mammary epithelial cell line, transcription of the β-casein gene was induced synergistically by insulin, glucocorticoid, and PRL (Doppler et al., 1990). The interaction of PRL with its receptor caused a rapid induction of the β-casein gene promoter-linked chloramphenicol acetyltransferase (CAT) activity in transfected cells. Dexamethasone increased the sensitivity of CAT activity in the cells in response to PRL, and the increase in CAT activity could be rapidly reversed by withdrawal of dexamethasone. Using primary mammary cell culture, Sasaki and Enami (1996) observed that additions of PRL and dexamethasone to the insulin-supplemented basal medium maximally stimulated the accumulation of γ-casein mRNA.

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Other hormones such as placental lactogen, growth hormone (GH), progesterone and thyroid hormones have also been demonstrated to be involved in the regulation of lactation. Experiments utilizing human placental extracts gave the first evidence that placental tissue was capable of producing PRL-like activity (Talamantes et al., 1980). Extracts from cotyledon tissue of ovine and bovine stimulated lactogenesis in mouse mammary explant culture, which supports the view that the ruminant placenta also has lactogenic activity (Akers 1985). In dairy cows, milk production is significantly increased by GH injection. The effects of GH on lactation will be discussed in details later in this chapter. It is known that progesterone stimulates bovine mammary development, but inhibits lactogenesis. For example, progesterone suppressed normal periparturient onset of synthesis of lactose, α-lactoalbumin, and casein as well as PRL-induced synthesis of milk constituents (Kuhn 1969; Goodman et al., 1990). Thyroid hormone was found to stimulate milk synthesis from the earlier experimental data of Cowie (1969), who found that hypophysectomy caused a rapid decline in daily milk yield of goats. Administration of glucocorticoid, triiodothyronine and GH could partially restore lactation. More recently, other researchers (Davis et al. 1988; Vonderhaar et al., 1986) reported that thyroxine injection increased milk production.

In summary, it has been known that hormones play a dominant role in controlling mammary development and lactation, but their complicated interactions and regulation of milk protein gene expression are not fully understood, and deserve further studies.

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1.2.3. Hormonal Regulation of Lactation between Rodents and Ruminants

Early researchers have noted species differences in hormone requirements for lactation. An ergotoxin, bromocriptine, completely inhibited mammary gland development and lactation in sows and rats (Flueckiger 1978). It was found that this drug acts by inhibiting PRL secretion, as was supported by the observation that the inhibition was reversed by injection of PRL in rats and rabbits (Zeilmaker and Carlsen 1962). Recent work in rats confirmed these findings and showed that inhibition of PRL secretion by bromocriptine injection reduced litter weight by 50% (Barber et al., 1992). These data further support that PRL plays a crucial role in rodent lactation.

In comparison, the suppression of PRL secretion by bromocriptine had no effect on established lactation in cows and goats (Kara et al., 1972; Hart, 1973). Once lactation is established, the level of circulating PRL and release of the hormone by milking can be reduced to a very low level without affecting milk yield in ruminants (Hart 1974; Smith et al., 1974). In bromocriptine-treated preparturient goats, PRL concentration was significantly depressed by the treatment and lactation performance was not affected (Forsyth and Lee, 1993). In addition to the differences in response of milk production to PRL between rodents and ruminants, the plasma level of PRL surge induced by milking is also different. For example, plasma PRL increases from 10 to 800 ng/ml in response to sucking in lactating rats while it only increases from 30 to 120 ng/ml in response to milking in lactating cows (Tucker 1971; Arbogast and Voogt 1996).

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The PRL reduction on lactating cows was not entirely unexpected as it had been known that once lactation had been restored in hypophysectomized goats, milk secretion could be maintained for several weeks in the absence of PRL by injection of bovine GH, triiodothyronine and dexamethasone (Cowie, 1969). PRL concentration in cows as well as in other ruminants is influenced by day length, temperature and consequently on seasons. Secretion of PRL is only slightly affected by stage of gestation so that in temperate climates the pattern of PRL in pregnancy is quite different in animals calving in autumn verses spring. Cows that calve in autumn experienced higher basal PRL concentrations in the second half of their pregnancy than cows calving in spring (Forsyth, 1996). It was also found that plasma PRL baseline was increased by long photoperiod (Bocquier and Oka, 1989).

In ruminants, GH is a potent lactogenic and galactopoietic hormone and is released during suckling or milking in goats (Hart and Flux, 1973). It has been known for many years that GH administration has a dose-dependent effect in stimulating milk yield in dairy cattle. The injection of cows with recombinant bovine GH increases milk yield by about 10-15% on average, with some studies showing much higher responses (Reviewed by Burton et al., 1994). However, GH action on rodent lactation is controversial. It is known that GH does not stimulate lactation in rodents because serum GH declines after suckling in rats, dogs and human (Tsushima et al., 1971). And inhibition of GH action by injection of anti-serum to rat GH in lactating rats did not have any effect on milk production, as measured by litter weight (Flint et al., 1992). On the other hand, GH can restore milk secretion in anti-GH/bromocriptine treated rats and local treatment with GH stimulates milk secretion (Flint et al., 1992).

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Apparently, there are differences between ruminants and rodents in the hormonal requirements for maintenance of lactation. PRL is a major hormone for lactation in rodents, but it is less important for maintenance of lactation in dairy cattle. It is more likely in dairy cattle that GH substitutes for the role of PRL in rodents. However, the characterization of rodent lactation as PRL-responsive and bovine as GH-responsive is not absolute. In mouse mammary explants, GH at 250 ng/ml did not induce lobulo-alveolar development, but at 1µg/ml did induce lobulo-alveolar development and β-casein mRNA synthesis (Plaut et al., 1993). The inhibition of PRL secretion in lactating rats alone reduced litter weight by 50%, but blocking of both PRL and GH actions completely inhibits milk synthesis (Barber et al., 1992). PRL and GH might interact in the modulation of lactation.

1.2.4. Growth Factors

The capacity for milk synthesis is determined by the number of secretory cells and the synthetic capacity per cell in the mammary gland. Following the discovery of various growth factors, experimental evidence has indicated that growth factors play important roles in modulation of mammary differentiation and function by complementing or mediating lactogenic hormonal actions. A growth factor may be produced locally in the mammary gland, therefore acting in a paracrine or autocrine manner, or alternatively it may originate in other tissues, acting in an endocrine manner.

1.2.4.1 Insulin-like Growth Factor-I

Insulin-like growth factor-I (IGF-I) is a peptide consisting of 70 amino acid residues in a single chain with three disulfide bridges. It is a potent mitogenic factor. It

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is believed that GH regulates lactation by stimulating liver or mammary IGF-I secretion in lactating animals (Van Wyk et al., 1974). Baumrucker and Stemberger (1989) employed mammary slice culture from prepartum and lactating cows and found that the IGF-I stimulated DNA synthesis by increasing incorporation of thymidine in the duct-epithelial, secretory alveolar epithelial and myoepithelial cells. *In vivo* studies found that close arterial infusion of IGF-I or IGF-II via the external pudic artery in the mammary gland increased milk yield and mammary blood flow in goats. The effect was rapid and became significant within 2 to 4 h and was confined only to the infused gland, suggesting a direct action of IGF-I on the mammary gland (Prosser et al., 1989; Prosser et al., 1994). Administration of GH to lactating cows increased the circulating level of IGF-I and milk yield in lactating cows. It was speculated that increased milk yield by GH injection is via IGF-I action in the mammary gland (Davis et al., 1987; Prosser et al., 1989; Cohick et al., 1989). IGF-I receptors were identified in the membranes of mammary tissue from lactating cows and ewes by crosslinking of [^{125}I] IGF-I to mammary cell membranes (Dehoff et al., 1988; Disenhaus, et al., 1988). Recent studies indicate that IGF-I acts as a survival factor, distinct from its mitogenic activity, to inhibit c-Myc- and etoposide-induced apoptosis in fibroblasts and BALB/C 3T3 cells (Harrington et al., 1994; Sell et al., 1995). It is possible that IGF-I could increase epithelial cell survival to stimulate milk synthesis in the mammary cell.

1.2.4.2. Epidermal Growth Factor

Epidermal growth factor (EGF) is a polypeptide consisting of 53 amino acid residues. It is found in the submandibular gland and has a variety of biological actions, including stimulation of mammary cell proliferation and modulation of cell function.

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Proliferation of mammary epithelium in explants, whole organ and cell culture is stimulated by EGF (Turkington 1969; Tonelli and Sorof, 1980; Yang et al., 1980; Taketani and Oka, 1983). Primary culture of bovine mammary cells was also sensitive to EGF. EGF stimulated cell proliferation when epithelial cells were cultured on collagen for 12 days in the presence of 3% fetal bovine serum and IGF-I. The expression of milk protein genes such as β-casein, κ-casein and α-lactoalbumin in cells cultured with PRL, insulin and hydrocortisone, was inhibited by addition of EGF (Topper and Freeman 1980). It was suggested that EGF played a role in the development of the mammary gland by stimulating early cell growth and suppressing precocious production of milk components (Vonderhaar and Nakhasi, 1986).

An *in vivo* study on EGF has shown that it is involved in the regulation of mammary development and lactation. Virgin mice were adenectomized to reduce the circulating concentration of EGF. Total mammary tissue mass of sialoadenectomized mice was less in late pregnancy and these animals produced less milk during lactation compared with that of sham-operated mice. The amount of milk was insufficient to nourish their pups and resulted in an increased incidence of pup mortality (Okamoto and Oka, 1984). Clearly, EGF is a potent effector for mammary development and cell proliferation during lactation; however, the mechanism through which EGF inhibits mammary cell milk synthesis is not well established.

Other growth factors such as transforming growth factor (TGF)-α and-β are also known to regulate mammary cell differentiation. TGF-α, a 50 amino acid polypeptide, when added to mice mammary explant culture together with insulin, PRL and steroid hormone, was able to promote lobulo-alveolar development (Snedeker et

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al., 1991). The mitogenic response of bovine and ovine mammary epithelial cells to EGF was additionally enhanced by TGF-α supplementation (Todaro et al., 1981). TGF-β1 has a biphasic effect on these cells, whereas relatively high concentrations (0.5-5 ng/ml) inhibit colony formation, lower concentrations (5-100 pg/ml = 200fM-4pM) stimulate colony elongation and branching (Soriano et al., 1996).

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1.3. GENE REGULATION AND SIGNAL TRANSDUCTION

It is clear that extracellular signals from steroid hormones as well as peptides and growth factors play essential roles in the control of mammary gland development and function. The signal transduction pathways for most lactogenic hormones are known to some extent. The majority of studies devoted to understanding signaling pathways in mammalian cells have primarily focused on transcriptional regulation. In order to outline the general understanding of gene regulation, the following provides a brief introduction of our current understanding of gene control mechanisms in eukaryotic cells, followed by the signal transduction pathways of the hormones and growth factors related to lactation.

1.3.1. Gene Regulation

The transcription of individual genes is switched on or off in eukaryotic cells by RNA polymerases as well as gene regulatory proteins. Eukaryotic RNA polymerases can not initiate transcription on their own. They require a set of proteins called general transcription factors, which must be assembled at the promoter before transcription begins. This assembly process provides multiple steps at which the rate of transcription initiation can be accelerated or decelerated in response to gene regulatory proteins, more commonly termed distal or proximal transcription factors (Roeder, 1991). The general transcription factors for RNA polymerase II are known to include transcription factor (TF) IID, which binds to TATA sequence (a short double-helical DNA sequence primarily composed of T and A nucleotides), TFIIE, TFIIF and TFIIH (White and Jackson, 1992). Many regulatory transcription factors are thought to facilitate (positive control) or hinder (negative control) the assembly process. It is a

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unique characteristic of eukaryotic cells that most transcription factors can act even though they are bound to DNA thousands of nucleotide pairs away from the promoter that they influence. Therefore a single promoter for a gene in mammalian cells can be controlled by many regulatory sequences scattered along the DNA and their respective specifically binding transcription factors (Johnson et al., 1989).

The complexity of gene regulation in mammalian cells is not only characterized by the involvement of many regulatory proteins in transcription initiation, but multiple steps of regulation in the pathways from DNA to RNA to protein. There are many steps in the pathway leading from DNA to protein, including transcription initiation, RNA processing, RNA transport and translation, and protein activation or secretion. In principle, all of these steps can be regulated in response to cellular regulatory signals (Darnell 1982). There is evidence from eukaryotic genes that initiation of transcription can be controlled. Differential splicing of primary RNA transcripts is observed in some genes such as immunoglobulins (Rogers et al., 1980; Maki et al., 1981). Gene expression can be further regulated by selecting which complete mRNA in the cell nucleus are transported to the cytoplasm, and consequently selecting which mRNA are translated into protein by the ribosome. In mammary cells, β-casein is also regulated by its mRNA stability. When mammary explants were cultured without PRL for 48h, they lost 95% of β-casein mRNA; however when given PRL, the half-life of β-casein mRNA increased by 20-fold (Guyette et al., 1979).

Gene expression is a complicated process that is still not well understood. Although multiple levels of gene control mechanisms are theoretically possible and

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have been studied to some degree, in most genes transcriptional controls have been speculated to be paramount. On the basis of the understanding of gene transcription, it is believed that once transcription has occurred, all other steps of gene expression follow and the appropriate protein is produced in a tissue-specific manner. This makes sense because, of all the possible controlling points, only transcriptional control ensures that no superfluous intermediates are synthesized so that the biological function of a cell will be more efficient in response to extracellular signals (Darnell 1982). However, in some cases the post-transcriptional control such as RNA splicing, mRNA transport and translational control also seems important controlling mechanisms for final protein synthesis as more information are obtained in this area.

1.3.2. Signal Transduction

In general, all the steroid hormones including glucocorticoid and progesterone act by a similar mechanism of signal transduction pathways in mammalian cells. Steroid hormones diffuse directly across the plasma membrane of target cells and bind to intracellular receptor proteins. These receptors are latent transcription factors, which upon binding steroids are capable of translocation from cytoplasma to nucleus and interacting with specific recognition sequences within target gene promoters. In its inactive state, the receptor is bound to an inhibitory protein complex that contains a heat-shock protein called Hsp90. The binding of ligand to the receptor causes the inhibitory complex to dissociate, thereby activating the receptor by exposing its DNA-binding site (Yamamoto 1985). It is thought that all of the intracellular receptor proteins bind to DNA as either homodimers or heterodimers. The glucocorticoid receptor recognizes a 15-bp DNA element (5'TGTACAGGATGTTCT 3'), which can

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also be bound by the progesterone receptor. There is not much known about what specific genes are actually regulated by steroid hormones in their target cells even though most steroids regulate wide ranges of cellular function (McDonnell 1995). In many cases the response takes places in two steps: the direct induction of the transcription of a small number of specific genes within about 30 minutes, known as the primary response; the products of these genes in turn activating other genes and producing a delayed secondary response (Evans 1988).

The extracellular signals from peptide hormones and growth factors are transduced by their respective cell-surface receptors. There are three known classes of cell surface receptor signaling proteins: ion-channel-linked, G-protein-linked and enzyme-linked. The receptors for most peptide hormones and growth factors involved in regulation of lactation are enzyme-linked cell surface receptors. They either act directly as enzymes, or are associated with enzymes; the enzymes are usually protein kinases that phosphorylate specific proteins in the target cells. Through a cascade of highly regulated protein phosphorylation, elaborate sets of interacting latent transcription factors relay most signals from the cell surface to the nucleus (Nishizuka, 1992). To activate or repress transcription, transcription factors must bind to a DNA sequence, and interact with the general transcription apparatus.

Among the receptors for hormones and growth factors in control of the mammary gland, insulin, EGF and IGF-I receptors are recognized to have tyrosine-specific protein kinase activity. These receptors are transmembrane, and the intracellular tyrosine kinase domain is activated when the hormone binds to the receptor. Once activated, the receptor transfers a phosphate group of the ATP to a

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selected tyrosine side chain on the receptor protein to initiate the intracellular signaling cascade (Carpenter, 1987). Receptor tyrosine kinases usually cross-phosphorylate themselves on multiple tyrosine residues, which then serve as docking sites for a number of intracellular signaling proteins via their Src homology region 2 (SH2) domains. Once bound, many of these proteins become phosphorylated, and are thereby activated to relay the signal into the cellular interior. Different receptor tyrosine kinases bind different combinations of these signaling proteins, and therefore result in different cell responses (Ullrich and Schlessinger, 1990).

The receptors for PRL and GH, as well as cytokines such as interferons, erythropoietin, interleukins and granulocyte colony-stimulating factor, work in a slightly different way. These receptors do not contain the catalytic activity for tyrosine kinase or a consensus ATP binding sequence (Stahl and Yancopoulos, 1993). Many receptors in this category work through associated tyrosine kinase, namely non-receptor tyrosine kinase, which could phosphorylate various target latent transcription factors when the receptors bind their ligands. There are at least two protein kinase families associated with these receptors: Src family and Janus family (Argetsinger et al., 1993; Mustelin and Burn 1993).

In the case of receptors associated with Janus family, the peptide hormone and growth factors bind to their respective receptors, and activate associated tyrosine kinases that phosphorylate various target proteins following the ligand binding. The activated Janus kinases primarily target activation of latent transcription factors by protein phosphorylation, causing translocation of the transcription factors from cytoplasma into nucleus in the cell. It is presumed that the phosphorylation process

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facilitates latent transcription factors interaction with the general transcription apparatus, however, the mechanism remains poorly understood (Hill and Treisman, 1995).

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1.4. TRANSCRIPTION FACTOR STAT5

It is known that PRL acts on mammary gland through a cell surface receptor. It was not delineated how the receptor signal was transduced to the milk protein genes in the mammary gland until the recent discovery of transcription factor Stat5. Stat5 stands for signal transducer and activator of transcription 5. It was identified in the mammary gland, and originally named "mammary gland factor (MGF)". The name Stat5 will be used throughout this thesis since it is more commonly used in the current literature.

1.4.1. Stat5 in the Mammary Gland

Recently major progress has been made in identification of transcription factors for β-casein gene expression. About 200 nucleotides located 5' of the RNA initiation site in the β-casein promoter were shown to be sufficient to confer hormonal response in transfected mammary HC11 cells (Doppler et al., 1989). The upstream 200-nucleotide promoter sequence in the β-casein gene is critical for effective gene transcription. The β-casein gene with the upstream 200-nucleotide promoter sequence was also expressed in the transgenic mice and transfected mammary cell lines (Roberts, et al., 1992; Rosen et al., 1989). Bandshift and DNA footprinting experiments, which are the two commonly used methods for studying DNA-protein interaction of gene promoter and transcription factors, revealed a multitude of factors able to specifically recognize this DNA sequence. Stat5 was found to bind to a DNA sequence in the promoter, and the DNA binding sequence at bout −80 to −100 nucleotide gave gave a particularly strong signal for Stat5 protein binding (Schmitt-Ney et al., 1991).

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The Stat5 DNA binding sequence serves the basis for detecting Stat5 DNA binding activity. Mammary nuclear extracts are mixed with the radioactively labeled DNA probe and then separated on electrophoresis. The DNA bound with Stat5 protein migrates more slowly than the DNA probe alone during electrophoresis. The amount of active Stat5 binding to DNA probe is proportional to the amount of radioactivity of the slowly migrating DNA probe; thus Stat5 DNA binding activity is determined in the nuclear extracts. Little or no Stat5 DNA activity was detected in early pregnancy, while the level of Stat5 was high toward the end of pregnancy and during lactation (Schmitt-Ney et al., 1992a). When lactation in rodents was terminated by removal of the pups, Stat5 activity decreased to an undetectable level within 24 h. However, within hours Stat5 activity returned to its maximal level by restoration of the pups to the dams (Schmitt-Ney et al., 1992b). The change of Stat5 activity is believed to be PRL-responsive since one of the most important consequence of suckling stimulus of pups is the maintenance of a high circulating level of PRL in rodents during lactation. Furthermore there is a difference in Stat5 activity response to pups removal between mice in early and late stage of lactation. The mammary glands from maximally lactating mice at 16 days postpartum contained dramatically reduced Stat5 activity after removal of the pups for only 8 h in comparison with early lactating mice, in which the down-regulation of Stat5 by pup withdrawal for 48 h was not pronounced (Schmitt-Ney, et al. 1992b). It is not clear why there is a difference in the response of Stat5 activity to pups' suckling between early and late lactation. It may be related to a slow response of prolactin to suckling in the late lactation in rodetns. In murine mammary epithelial cells, little Stat5 activity was detected in the nuclear

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extract when cells were cultured in the absence of lactogenic hormones PRL, dexamethasone, and insulin. Treatment of the HC11 cells with the lactogenic hormones led to a strong induction of Stat5 activity (Schmitt-Ney et al., 1992a). The Stat5 DNA binding sequence was identified to the region between nucleotide -80 to – 100 of the β-casein gene promoter by DNA footprinting. When mutations were introduced into the Stat5 binding oligonucleotide sequence, Stat5 activity and β-casein gene transcription was not detected in transfected HC11 cells. Two specific nucleotides in the core region between-100 and –85 of the β-casein promoter were mutated from GACTTTCTT*GG*AATTAA to GACTTTCTT*TT*AATTAA and tested in transfected HC11 cells. It was found that the activity of Stat5 binding to the mutant sequence was reduced by 100-fold. Lactogenic hormones treatment of HC11 cells, transfected with the mutated promoter-chloramphenicol acetyltransferase (CAT) construct, failed to induce CAT activity, indicating Stat5 binding is necessary for β-casein gene transcription (Schmitt-Ney et al., 1992a).

Genomic clones of several caseins from various species have been isolated and characterized. There are several common sequences in the upstream promoter regions of these genes, including the Stat5 binding sequence 5'-TTCTTGGAATT-3'. This sequence is highly conserved at similar positions, in the 5'-flanking regions of at least six different casein genes from mice, rats and cattle (Yoshimura and Oka, 1989; Gorodetsky et al., 1988). The conservation of a regulatory sequence for Stat5 binding is suggestive of functional roles of the DNA motif and Stat5 binding in the transcription of milk protein genes. The binding sequence of Stat5 was also characterized in the ovine β-lactoglobulin gene in transgenic mice and primary

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mammary cells. Three Stat5 binding sites were identified in the promoter regions of β-lactoglobulin. Abolishing binding to a single Stat5 site had no effect on induction of β-lactoglobulin expression in response to the lactogenic hormones PRL, dexamethasone and insulin. However, the disruption of two Stat5 sites abrogates induction, and mutation of all three elements completely abolished hormonal responses. These results were confirmed in transgenic mice (Burdon et al., 1994a and 1994b), where mutagenesis of the binding sites in sheep β-lactoglobulin gene promoter region reduced expression of β-lactoglobulin gene in lactating mammary glands.

CCCT AGAATTTCTTGG	TTCTTGGAATTGAA	AACCACAAAATTAGCATGACA	mouse β-casein
- - CTC AGAATTTCCⳑAG	TTCTTAGAATTTAT	AACCACAAAATTAGCATGACA	rat α$_{s1}$-casein
CCCC AGAATTTCTTGG	TTCTTGGAATT AA	GAACCAC AAATTAGCATGTCA	rat β-casein
CCTT AGAATTTCTTGG	TTCTTAGAATTTAA	AAACCACAAAATTAGCATTTTA	bovine α$_{s1}$-casein
CCCC AGAATTT TTGG	TTCT AGAATTTCA	AAACCACAAAATTAGCATGCCA	bovine β-casein
* * * * * * * * * ** * * * *	* * * * * * ** * *	* * * * * * * * * * * * * * *	
Block A	Block B	Block C	

CCCT AGAATTTC-TGG TTCTT--GAATT--AA AAACCACAAAATTAGCAT—TCA

Fig.1.1. Conserved sequences in the 5' flanking regions of casein genes.

Three highly homologous sequences which were identified by homology search are designated as Block A, B, and C. The consensus sequences of these homologous regions are shown (adapted from Yoshimura and Oka 1989; Gorodetsky et al., 1988).

1.4.2. Stat5 and Signal Transduction

The gene encoding Stat5 has been cloned and sequenced (Wakao et al., 1994). Stat5 consists of 734 amino acids and is characterized by a src homology 2 (SH2)-like domain and a src homology 3 (SH3)-like domain and a putative tyrosine phosphorylation site at position 694 in the amino acid sequence. The sequence analysis of Stat5 has revealed that it belongs to the molecular family of **signal transducers and activators of transcription (Stat).** The Stat proteins are characterized by phosphorylation on tyrosine and dimerization by reciprocal SH2 phosphotyrosine interaction, resulting in translocation into the nucleus to regulate transcription of many different genes (Darnell et al., 1994; Darnell 1997). They are recognized as ligand-induced transcription factors in cells and tissues exposed to many cellular polypetide signals (Darnell et al., 1994; Schindler et al., 1995; Leaman et al., 1996). This group of proteins is implicated in a wide variety of biological events. Three known Stats have a narrow activation profile: Stat2 is activated only by interferon-α. Stat4 in lymphocytes is activated only by interleukin-2 and interferon-α and Stat6 is activated only by interleukin-4 and interleukin-3 (Darnell 1997). Originally Stat1 was identified as interferon-α and-γ-induced and Stat3 was activated by interleukin-6 (Shuai et al., 1992; Fu et al., 1992; and Zhong, et al., 1994). Presently it is known that Stat1 and Stat3 are activated by many different cytokines (Darnell 1997).

Stat5 is one of the Stat proteins involved in the signal transduction of multiple hormones and growth factors. The following review will focus on the mammary gland and its regulation by lactogenic hormones and growth factors. Groner and colleagues

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conducted a series of experiments using COS cells co-transfected with cDNA encoding Stat5 and a corresponding hormone or growth factor receptor. They found that PRL treatment of the transfected cells caused activation of Stat5 within 5-10 minutes. This activation did not require ongoing protein synthesis and tyrosine kinase inhibitor genistein prevented Stat5 activation. Using a mutant Stat5 cDNA, which has a phenylalanine replacement at position 694 (tyrosine), they showed the loss of tyrosine phosphorylation and Stat5 activation by PRL in the transfected COS cells. This result indicated that phosphorylation of tyrosine at position 694 in the Stat5 DNA sequence is essential for activation by PRL (Gouilleux et al., 1994). In vitro, Stat5 was phosphorylated by Janus kinase 2 (Jak2) and the phosphorylated Stat5 exhibited DNA binding activity, but was not phosphorylated by fyn, lyn and lck tyrosine protein kinases. More recently, Stat5 (both a and b forms) was phosphorylated by src/abl kinase, but only the phosphorylated Stat5b was able to translocated into cell nucleus. Jak2 appears to be an important protein kinase for PRL induction of Stat5 activation (Kazansky et al., 1999).

Similarly, when the cDNA encoding receptor for GH, erythropoietin or granulocyte-macrophage stimulating factor was respectively cotransfected into COS cells with Stat5 cDNA. Stat5 DNA binding activity was induced with the corresponding hormone addition. It was shown that Stat5 activation by erythropoietin and GH required the phosphorylation of tyrosine residue 694 of Stat5 (Gouilleux, et al., 1995). The transcriptional induction of a β-casein promoter luciferase construct in the transfected COS cells was only observed in PRL activation of Stat5, but not in the erythropoietin and GH-treated cells. It was not known why activated Stat5 by GH and

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erythropoietin in the transfected cells did not result in expression of β-casein promoter luciferase. It might indicate that efficient β-casein promoter activation during GH or erythropoietin induction requires Stat5 as well as some other transcription factor interactions with the promoter in the transfected COS cells.

In transfected mammalian cells, it was also found that interleukin-2, -3 and–5 signals were transduced through Stat5 (Mui et al., 1995; Wakao et al., 1995). In mouse liver, EGF can induce tyrosine phosphorylation and nuclear translocation of Stat5 (Ruff-Jamison et al., 1995).

Hennighausen and colleagues identified two Stat5 genes, Stat5a and Stat5b. On the protein level, mouse Stat5a and Stat5b show 96% sequence similarity (Liu et al., 1995). Transient differentiation of mammary alveolar cells and milk protein gene expression during estrus in virgin mice coincided with transient Stat5a and Stat5b phosphorylation. In transgenic mice expressing the cytoplasmic portion of the *Int 3* gene, the mammary alveolar development and function are impaired, and the β-casein gene was expressed at a very low level. Mammary Stat5a and Stat5b phosphorylation and dimer formation were also reduced in these mice. These results indicate that both Stat5a and Stat5b phosphorylations are linked to mammary differentiation (Liu et al., 1996).

By transgenic technology, Stat5a and Stat5b knockout mice have been created. Each mutant shows a highly specific phenotype. Stat5a-deficient animals (Stat5a$^{-/-}$) develop normally and were indistinguishable from hemizygous and wild-type littermates in size, weight, and fertility. Stat5a$^{-/-}$ females did not show mammary lobuloalveolar outgrowth during pregnancy and failed to lactate after parturition.

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Stat5b^{-/-} females can lactate, but Stat5b^{-/-} males lost male-characteristic body growth rates and male-specific live gene expression (Udy et al., 1997). These results document that Stat5a is the principal and obligate mediator of mammopoietic and lactogenic hormone signaling (Liu et al., 1996).

In the mammary gland, the basement membrane is necessary for the PRL-mediated control of milk protein gene expression (Liu et al., 1997; Barcellos-Hoff et al., 1989; Streuli et al., 1995). Using mammary cell culture derived from transgenic mice harboring the ovine β-lactoglobulin gene, it was shown that a basement membrane component, laminin-1, induced a high level of β-lactoglobulin synthesis in the presence of prolactin. The DNA binding activity of Stat5 was present only in extracts of mammary cells cultured on basement membrane (Streuli et al., 1995). This indicates that extracellular matrix components are also involved in PRL-activated Stat5 signaling pathways in the mammary gland.

It is clear from the accumulated evidence in transfected mammalian cells that PRL, GH and EGF induce Stat5 activation, and that the activation of Stat5 is essential for the function of mammary gland. In the upstream elements of these hormonal signal transduction pathways, Janus kinase 2 (Jak2), a 130-Kda protein has been identified to be one of the receptor associated tyrosine kinases. Binding of GH in rat-pre T lymphoma cell line (Nb2) activates Jaks (Argetsinger et al., 1993). In the mouse mammary explant and Nb2 cell culture, Jak2 was also activated by PRL, indicating that Jak2 is associated with the PRL receptor and serves as an early and initial signal molecule for PRL (Campbell et al., 1994).

A working model for the Stat5 signal transduction pathway was hypothesized as follows. Following ligand binding, membrane-bound hormone or cytokine receptor activates Jak2. This activation leads to tyrosine phosphorylation of the Jak2 molecule itself. The activated Jak2 converts inactive Stat5 into its active form through tyrosine phosphorylation. The tyrosine phosphorylation of Stat5 results in the ability of the protein to form homodimers via the SH2 phosphotyrosyl interaction, followed by Stat5 translocation and DNA binding activity to influence milk protein gene expression in the nucleus (Gouilleux et al., 1994; Wood et al., 1995.) This is shown schematically in Fig.1.2.

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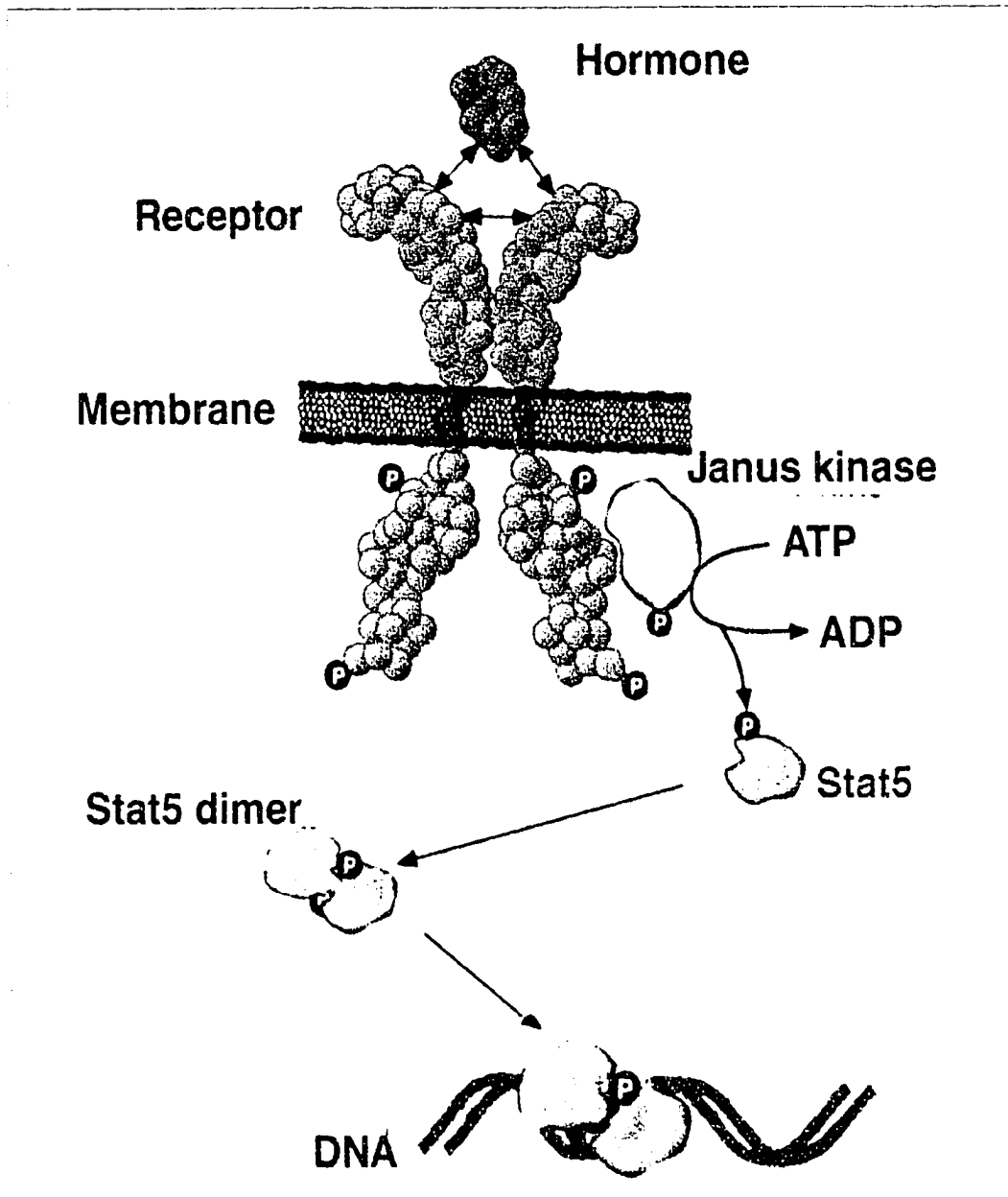
Fig. 1.2. Model of Stat5 signal transduction pathway

Binding of hormones to their respective receptors activates the receptor associated protein kinase Jak2. This kinase catalyzes the tyrosine phosphorylation of Stat5. Stat5 then becomes activated and translocates into the nucleus where it binds specifically to the DNA sequence in the promoter regions of milk protein gene, thus influencing their gene transcription. Adapted from Wood et al., 1995.

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1.5. NON-HORMONAL INFLUENCES OF LACTATION

Hormones and growth factors play an important role in control of the mammary gland. Milk production is also influenced by other physiological factors such as milking frequency, nutrition, genetics, lactation stage and environment. The mechanisms of these factors in the regulation of lactation are mostly not explicit. Although they are probably involved in multiple levels of interactions among hormonal and non-hormonal actions on mammary gland, the following section will focus on their non-hormonal influences on lactation.

1.5.1 Milking Frequency

Milk production is related to milking frequency. In the dairy cow, milking is normally performed twice daily at intervals between 12:12h and 16:8h. Milking three times or four times daily increases yield relative to twice daily milking (Poole 1982; Hillerton et al., 1990 and Bar-peled et al., 1995). The increase in milk yield is about 10 to 15% over twice daily milking. In contrast, less frequent milking reduces milk production. Once daily milking reduces milk yield as much as 20% in the first week in comparison with twice daily milking (Knight et al., 1994).

It has been shown that the effects of milking frequency on milk production are exerted through mammary local control. A low molecular weight protein was identified in goats' milk. This protein is able to reduce the rate of milk secretion by feedback inhibition. This protein is termed feedback inhibitor of lactation (FIL). It decreases milk secretion when introduced into a mammary gland of lactating goats (Wilde et al., 1995). When lactating goats were immunized against FIL, the antibody to FIL was consistently detected in milk and the rate of decline in milk secretion was

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significantly reduced compared with sham-immunized controls (Wilde et al., 1996), giving further evidence of the presence of FIL in the milk. These results suggest that milk contains an inhibitor of its own secretion which accumulates during storage within the lumen of the mammary gland and acts in an autocrine manner on the secretory cells (Wilde et al., 1998).

1.5.2. Nutrition

Amino acids are the building blocks for protein and are essential for maximal protein synthesis. The synthesis of milk protein in the mammary gland is also influenced by other factors such as energy supply. The response of milk protein to dietary protein and energy supply is complicated by rumen microbial fermentation. For example, an increase in the concentrate portion of the diet will generally result in increased energy intake and nutrient supply to the mammary gland. The type of concentrate, ratio of concentrate to forage and method of grain processing have great impact on rumen carbohydrate fermentation, which, in turn, may influence microbial growth and patterns of volatile fatty acids production. This will affect the energy and amino acid supply to the mammary gland. Furthermore, microbial protein, as well as undegraded feed protein, is important sources of amino acids. The concentration and degradability of dietary protein may affect microbial growth, the proportion of microbial protein and undegraded feed protein entering the duodenum, and the energy intake.

Nutritional trials have been conducted to define nutrient requirement and feeding regime for optimal milk protein production. Supplements of rumen-protected protein sources in the ration, which could escape rumen degradation by heat, extrusion

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and chemical treatment, will increase the supply of amino acids to the mammary gland for lactating cows. When protein degradability was altered by employing protein supplements that supplied approximately 50% of the total dietary protein, large effects were observed on the profiles of amino acids in the digesta reaching the duodenum (Stern et al., 1983; Santos et al., 1984). Progressive increases in dietary nitrogen intake via corn-gluten meal increased the total nitrogen flow to the small intestine, resulting in an increase in milk protein from 3.15 to 3.31% without changing in milk yield (Stern et al., 1983).

In a study with an inadequate initial crude protein level, 26% and 40% increases in yields of milk and milk protein were respectively achieved by supplementing 9% crude protein diet with formaldehyde treated casein (Broderick et al., 1974). However, many studies with cows consuming adequate amounts of crude protein showed little or no milk protein response to ruminally undegradable protein or amino acid supplementation (Reviewed by DePeters and Cant, 1992). In commercial herds, fish meal supplementation (low rumen protein degradability) in early lactation increases milk yield approximately 9%, but did not affect protein content (Miller et al., 1981). Altering the rumen degradability of dietary protein content by various treatment methods (heat, extrusion, or formaldehyde) did not result in milk protein changes (Mielke and Schingoethe 1981). In some studies, milk protein content was decreased by increased supplies of undegradable protein sources (Forster et al., 1983; Schingoethe et al., 1988).

Feeding lactating cows with rumen-protected amino acids to improve milk and milk protein yield is based on the concept that some amino acids may be limiting for

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mammary milk protein synthesis. The response to feeding individual amino acids is not consistent. Feeding protected methionine increased milk yield, milk protein content, and protein yield in lactating cows (Illg et al., 1987). It was shown that addition of rumen-protected methionine to the high protein diet could increase milk protein concentration and yield linearly. Milk yield, dry matter intake and milk fat content were unaffected. Each gram of methionine increased milk protein yield by 4 g, while milk protein percentage increased from 2.89 to 2.99 with the addition of 10.5 g/day of methionine (Armentano, et al., 1997). In other studies, protected methionine did not influence milk or milk protein yield. A series of experiments conducted in South Dakota measured milk production in response to the addition of 15 g of methionine to a diet containing various heated and unheated soy proteins. Milk protein concentration increased 0.05% on average in three experiments (Yang et al., 1986; Casper et al., 1987; Schingoethe et al., 1988), but further study found no increase in milk protein concentration (Munneke et al., 1991). Therefore, there are considerable experimental variations in milk protein response to protected amino acids feeding.

Postruminal infusion of nutrients provides an experimental advantage over dietary feeding in studying the response of milk protein production to nutrients such as protein and amino acids. Positive results with respect to nutritional manipulation of milk protein have been obtained with postruminal infusion of casein. There is convincing evidence, from a large number of studies, that abomasal infusion of casein in lactating cows and goats can lead to increases in milk and milk protein yield (DePeters and Cant 1992). In a report with an extremely low crude protein diet, it was observed that milk protein yield increased by 40% and milk yield increased by 32%

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through abomasal infusion of casein in cows fed a 13% crude protein diet. Protein content in the milk increased from 3.01 to 3.18% (Whitelaw et al., 1986). In another study, the daily yield of milk nitrogen increased linearly as progressive amounts of casein were infused. Milk protein content increased from 2.8 to 2.94% and protein yield from 717 to 786 g/day with daily infusion of calcium caseinate from 177 to 762 g/day to supply dietary protein level from 90 to 130% of the requirement (Guinard et al., 1994c).

It was proposed that the response of milk synthesis to the infusion could be due to provision of limiting amino acids, gluconeogenic substrates, or changes in blood hormone profiles (Clark, 1975). Casein infusion tended to increase mammary uptake of energy precursors such as glucose, β-OH-butyrate and non-essential fatty acids. The mammary gland was upregulated to meet the demand for the synthesis of other constituents when milk protein is increased (Cant et al., 1993; Guinard et al., 1994a). Attempts to mimic the stimulatory effect of casein on milk yield and milk protein secretion by postruminally infusing soya-bean protein isolates reinforced with methionine and lysine failed (Choung and Chamberlain 1993). The precise mechanism of increased milk protein synthesis produced by postruminal infusion of casein remains unclear. It is suggested that biologically active peptides might be produced during digestion of casein, and they may have a pharmacological action on protein synthesis.

The availability of free amino acids to the mammary gland in relation to milk protein production has also been studied. The regulation of mammary blood flow and amino acid transport influences the rate of uptake to the mammary cells. In dairy

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nutrition, methionine and lysine are often implicated as the first limiting amino acids. There have been suggestions that branched-chain amino acids are most likely limiting for higher level of milk protein production in lactating cows (Chandler 1989; Mantysaari et al., 1989). It is known that the net uptake of leucine by the mammary gland exceeds its secretion in milk protein, and this differential becomes even greater when milk protein output is increased by duodenal infusion of casein (Guinard et al., 1994b). The fate of this additional leucine in the mammary gland of cows is unknown. The oxidation of leucine by the mammary gland has been positively correlated with dietary protein intake (Bequette et al., 1996a, 1996b; Oddy et al., 1988). In lactating goats, it was found that intravenous amino acid infusion reduced mammary oxidation of leucine to approximately one-third of the control value, without affecting milk protein synthesis. In the lactating sows, it was also found that the uptake of leucine, isoleucine, valine, phenylalanine, threonine and arginine significantly exceeded the output of these amino acids in milk. It was speculated that mammary retention of these amino acids might indicate their specific requirements at the mammary cellular level for such uses as structural protein synthesis and cellular remodeling (Trottier et al., 1997). Glutamate and glutamine are also known to be potentially limiting for milk protein synthesis in the high yielding dairy cow. It is found that their decreases in plasma and increases in mammary uptake exceeded most amino acids with stimulated milk synthesis in lactating cows. (Meijer et al., 1995; Lykos and Varga 1997).

1.5.2. Other Factors

Many factors other than nutrition affect milk and milk protein production, including breed, stages of lactation, environment, and mammary gland diseases. They

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generally influence milk protein synthesis in the mammary gland through complicated physiological processes. The protein content, like other components in the milk, varies greatly among species, for example, the content of milk protein ranges from 1-20% across the mammals. The milk from a black bear has 15% protein, reindeer 10%, cow 3% and human 1%. Within dairy cattle breeds, protein content can vary from less than 3% to about 4%. Greater variation is observed for milk fat with almost two-fold difference among breeds. The variation of milk composition among breeds of dairy cattle demonstrates the extent to which milk component synthesis in the mammary gland is under genetic control. The genetic correlation between fat and protein contents of milk is in the order of 0.55 on average in commercial dairy breeds (Gibson, 1989). Thus, there is a potential opportunity to selectively improve milk protein content, without likewise increasing fat content through animal breeding.

Milk yield dramatically increases in the early stage of lactation, reaches the maximum level within one to three months after calving, and then gradually decreases in the middle and late stages of lactation. The content of total nitrogen, casein and non-protein nitrogen decrease rapidly in the early stage (first three months) of lactation, which is followed by a gradual increase through to the end of lactation (Waite et al., 1956). During the first two months of lactation, the relative percentage of α_{s1} and α_{s2} - casein decreased, but β-casein increased with little change thereafter (Kroeter et al., 1985). A higher proportion of γ-casein was found in the milk in late lactation (7-8 months) than that of early lactation. Higher plasmin activity during late lactation was also observed and might be the cause of an increased γ-casein level from proteolytic destruction of β-casein (Korycka-Dahl et al., 1983). In the milk from goats,

the relative amounts of the breakdown products of plasmin attack on β-casein and γ-caseins were highly negatively correlated with milk yield in the declining phase of lactation. And the relative amount of kappa-casein increased by approximately 50% after peak lactation and its concentration almost doubled near the end of lactation (Brown et al., 1995).

The influence of environmental factors on milk composition is mediated by seasonal changes. High environmental temperature in the summer reduces total protein content of milk and may decrease the firmness of curd produced from such milk (Fegan 1979). DePeters and Cant (1992) compared 30 herds across four dairy breeds in California and found that total milk protein content was lowest in the summer and highest in the winter.

The predominant disease of mammary gland influencing milk protein is mastitis. Mastitis results in a reduction in casein content and an increase in whey protein content of milk on a relative basis. Proportions of β-casein and α_{s1}-casein both decreased but the proportion of κ-casein and other unidentified proteins increased with increasing leukocyte concentration in the milk (Munro et al., 1984).

1.6. SUMMARY

The biological function of mammary gland, characterized by milk synthesis and secretion, is influenced by hormonal as well as non-hormonal physiological factors. Lactogenic hormones are known to play an essential role in the initiation and maintenance of lactation. They have been studied in the mammary gland of rodents and *in vitro* tissue culture, and the models of their action are therefore established based on rodents. It is known that the PRL acts through transcription factor Stat5 to be transduced to cell nucleus. Stat5 is involved in the signal transduction pathways of multiple hormones and growth factors in addition to PRL in transfected mammalian cells. Non-hormonal factors, especially nutrition and milking frequency have been widely studied in dairy cattle production. Nutritional approaches to increasing milk protein concentration are limited by substantial variations and poor reproducibility in the observed experimental data, indicating that the lack of understanding of biological control of mammary gland is hindering the development of effective means of manipulation of milk composition.

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1.7. HYPOTHESIS

The activation of Stat5 is known to play a critical role in mammary function. The role of Stat5 in transducing prolactin signals and mammary development has been established in transfected COS cells and trangenic mice. Multiple hormones and cytokines induce Stat5 activity in non-mammary cells, and Stat5a deficient mice showed a failed lactation phontype. The critical role and function of Stat5 has not been widely studied in the bovine mammary cells. The research contained in this thesis was directed toward the investigation of Stat5 and its regulation in bovine mammary gland. Rat mammary glands were used as an experimental control.

Lactation in the bovine is characterized by the involvement of multiple hormones and hormonal interactions. In addition to PRL in regulation of lactogenesis and maintenance of lactation, GH and possibly IGF-I in mediation of GH action are galactopoietic in ruminants. In lactating dairy cattle, increased milk yield has been associated with GH infusion, and lactation is also regulated through other wide ranges of physiological influences in addition to hormonal treatment. For example, milk production is positively related to milking frequency, and the asssociated local changes are thought to play a role in upregulation of milk synthesis. **Based on the roles of Stat5 in hormonal signal transduction and mammary function, we hypothesized that Stat5 may serve as a common point in the signal transduction pathways of lactogenic hormones such as PRL, GH and IGF-I in bovine mammary cells *in vitro*. Furthermore Stat5 may also play the same role in connecting different pathways *in vivo* so that local changes resulted from different milk fequencies may influence Stat5 activity.**

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CHAPTER 2. ACTIVATION OF TRANSCRIPTION FACTOR STAT5

RESPONDS TO PROLACTIN, GROWTH HORMONE AND

IGF-I IN RAT AND BOVINE MAMMARY EXPLANT

CULTURE

2.1. INTRODUCTION

Lactation in the bovine is characterized by the involvement of multiple hormones and hormonal interactions. In most species, prolactin (PRL), in the presence of insulin and glucocorticoids, is the key factor in lactogenesis and maintenance of lactation. In ruminants, growth hormone (GH), and possibly insulin-like growth factor-I (IGF-I) in mediation of GH actions, are also galactopoietic (Tucker, 1981). Injection of recombinant bovine GH enhances the circulating levels of GH and IGF-I, and milk yield increases by about 10-15% on average in lactating cows during GH treatment (Bauman et al., 1985).

Each of these hormonal effectors has a specific cell surface receptor. Prolactin primarily acts through its long form receptor in mammary cells (Hennighausen, et al 1997). There is evidence that the mRNA and protein of GH receptor, as well as IGF-I type I receptor ligand binding and mRNA, are present in the mammary gland (Glimm et al., 1990; Ilkabahar et al., 1999; Rechler, 1985). These peptide hormones thus act via signaling pathways originating from their respective receptors, however since they act ultimately at a common site, milk protein gene expression, their signal transduction pathways must at some point interact or converge. It is not well understood how these pathways integrate to coordinately regulate cell function in the mammary gland.

Mammary cells contain a latent transcription factor acting on casein gene promoter sequences, known as mammary gland factor or signal transducer and

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activator of transcription (Stat)-5 (Wakao et al., 1994). Stat5 is implicated in prolactin–induced β-casein gene transcription in rodent cells. The signaling pathway of PRL is proposed to involve receptor binding, activation of Janus Kinase 2 (Jak2) and subsequent Stat5 phosphorylation by this kinase. Phosphorylation confers activity and nuclear translocation of the Stat5 (Gouilleux et al. 1994). Two isoforms of Stat5 (a and b) have been identified in mice. While Stat5a-deficient transgenic mice fail to lactate after parturition, Stat5b-deficiency results in abnormal male-specific gene expression in the liver (Liu et al., 1997; Udy et al., 1997). These results suggest that Stat5 may play a central role in controlling mammary function. In the bovine, where lactation regulation involves multiple hormonal influences, it is not known how Stat5 might intercede in the signaling pathway of these hormones.

We hypothesized that Stat5 may serve as a common point in the signal transduction pathways of lactogenic hormones in bovine mammary cells. Pathway convergence has been shown in growth factor / cytokine signal transduction. For example, interleukin-3, granulocyte-macrophage colony-stimulating factor, and mast cell growth factor all activate Jak2 in transfected COS cells (Brizzi et al., 1994). There is evidence from non-mammary cells that a number of hormonal signal transduction pathways may be interconnected at the level of Stat5 activation. Growth hormone as well as several cytokines in transfected COS cells are able to activates Stat5 (Ihle and Kerr, 1995). GH receptor has been shown to associate with Jak2 in transfected Nb-2 cells (Argetsinger et al., 1993), thus GH may act on the mammary gland through Jak2-Stat5 pathway. However, GH is also implicated in indirect actions on lactation by stimulating the secretion of IGF-I, which in turn regulates milk synthesis (Etherton

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and Bauman 1998). It is unknown whether IGF-I interacts with the mammary Jak2-Stat5 pathway.

We used mammary explant culture from lactating rats and cows in our studies to explore the effects of PRL, GH and IGF-I addition on Stat5 activation and protein level.

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2.2. MATERIALS AND METHODS

Animals

Studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care. Female Sprague-Dawley rats from a colony maintained at the University of Alberta and Holstein cows from the Dairy Research and Technology Center of the University were used. In both species, animals in mid-lactation were used. Rats were housed in individual wire mesh cages in temperature (24°C) and humidity (80%)-controlled rooms in a 12h light-12h dark cycle. Rats were fed laboratory chow containing 24% crude protein (Continental Grain, Chicago IL). Cows were fed a total mixed ration containing 50% concentrate and 50% forage and housed in tie-stalls with 24 h light.

Mammary Gland Explant Culture

Lactating rats were separated from their pups for 24 h prior to tissue sampling to reduce basal Stat5 activity. Rats were killed by CO_2 asphyxiation and the fourth mammary gland was dissected and removed aseptically. Mammary explant culture was carried out as described (Topper et al., 1975) with the following modifications. Mammary explants (1-2 mm thick and wide) were prepared with scalpel and fine scissors in medium 199 with Earle's salts (Gibco BRL, Geithersburg, MD). Mammary explants were cultured in medium 199, supplemented with 1μg/ml bovine insulin (Sigma, St Louis, MO), 1μg/ml hydrocortisone and with treatment hormones: ovine PRL (Sigma, St Louis, MO), GH or IGF-I (Gibco BRL, Geithersburg, MD). Rat and bovine GH were a gift from Dr. A. F. Parlow through the National Hormones and

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Pituitary Program, National Institute of Diabetes and Digestive and Kidney Disease

(Bethesda, MD). The hormones were added at 50ng/ml and 200 ng/ml for PRL and 50

ng/ml for GH or with the concentrations (1µg/ml) commonly used for differentiation

of explant cultures (Topper et al., 1975). IGF-I (human recombinant, Gibco BRL,

Geithersburg, MD) was added at 50 ng/ml in both rat and bovine mammary explant

culture. Explants were cultured in 14 ml sterile tubes at 37°C and bubbled with

5%CO$_2$ and 95%O$_2$.

Bovine mammary tissue was obtained by biopsy (Knight et al., 1992).

Mammary gland was biopsied from cows that had not been milked for 6 h (Fig. 8) and

22 h (Fig. 15). The period of withdrawal from milking was lengthened to 3 days in

later studies (Fig. 9,10,11) because our first studies showed that bovine mammary

Stat5 decreased more slowly than in rat after withdrawal of milking. Deep sedation of

the cow was achieved by slow intravenous injection of xylazine. Animals were tipped

and held recumbent on their right side. Local anaesthesia was achieved by a line block

with subcutaneous injection of 5-10 ml 2% lignocaine hydrocholoride. A 5-10 g

portion of secretory tissue was surgically removed. Haemostasis was achieved with

suturing. Milk yield of biopsied cows returned to normal levels within one week.

Explants were immediately prepared and cultured as described for rats except that

bovine GH was used to replace rat GH. Freshly isolated tissues as well as cultured

explants were frozen in liquid nitrogen and stored at -70°C until nuclear extraction.

Mammary explant cultures for Stat5 activation in response to the hormones were

repeated at least 2-3 lactating rats and cows, but only one representative Stat5 assay is

shown in the results.

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Preparation of Nuclear Extract

Mammary nuclear extracts were prepared as described (Standke et al., 1994) with the following modifications. All chemicals used were purchased from Sigma except quoted manufacturers. Tissue (1-2 g) was pulverized in liquid nitrogen using a pre-chilled (-70°C) mortar and pestle. The frozen and pulverized tissue was immediately transferred to a 14 ml Falcon tube, and minced in 2 ml lysis buffer (10 mM Hepes, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10 % glycerol, 5μg/ml aprotinin, 5μg/ml leupeptin, 2μg/ml pepsatin) in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Then 3-5 ml of the same buffer was added and the dispersed tissue was homogenized in a 7ml glass Dounce tissue grinder (Wheaton Scientific, Millville, NJ). The homogenates were centrifuged on a cushion of the lysis buffer at 100,000X g (Sorvall® Ultra Centrifuge, Dupont, Wilmington, DE) for 20 minutes using a Sorvall® TH 641 rotor.

Nuclei were precipitated through the cushion to the bottom of the centrifugation tube and the supernatant was discarded. The DNA yield (8-10 mg / g tissue) from the nuclear purification was determined using the method of Labarca and Paigen (1980) and was not different between any experimental treatments. The pellets were resuspended in hypertonic buffer containing 20mM HEPES (pH 7.9), 400mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 0.1% Trition X-100, 20% glycerol, 1mM dithiothreitol (DTT), 2 mM sodium orthovanadate, 0.2mM phenylmethylsulfonyl floride (PMSF), 5μg/ml leupetin, 5μg/ml aprotinin and 2μg/ml pepstatin for 20 minutes on ice. The extracts were then centrifuged at 14,000 rpm for 5

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minutes at 4°C. Nuclear extracts were frozen in liquid nitrogen and stored at -70°C. The protein content in the nuclear extracts were measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA) and used as a basis for sample loading in the assay of Stat5 activity and Western blots (see below). For the study of dephosphorylation of Stat5, nuclear extracts were isolated without using sodium orthorvanadate in the extract buffer and protein tyrosine phosphotase (PTP, human recombinant, Calbiochem, San Diego, CA) was used at the concentration of 1 unit/μg nuclear protein to dephosphorylate nuclear extracts. One unit of PTP is defined as the amount of enzyme that will hydrolize 1.0 nmol of p-nitrophenyl phosphate per minute at 30°C, pH 7.0.

Electrophoretic Mobility Shift Assay (EMSA)

Stat5 DNA binding activity was detected using electrophoresis mobility shift assay, in which a labeled double-stranded DNA sequence was used as a DNA probe to bind active Stat5 protein in nuclear extracts. The Stat5-DNA complex and the free DNA were separated using polyacrylamide gel electrophoresis and visualized by autoradiography. The Stat5 binding site (5′-AGATTTCTAGGAATTCAAATC-3′) of the bovine β-casein promoter was used to design the probe. This oligonucleotide was hybridized with its complimentary oligonucleotide (5′-GATTTGAATT-3′) at 55 °C for 10 minutes. The doubled stranded labeled DNA probe was obtained by fill-in reaction with [α-^{32}P]dATP (Amersham) using DNA polymerase I Klenow fragment (Pharmacia Biotech, Piscataway, NJ) at 37 °C for 1 h. The unincorporated radioactive dATP was removed by Sephadex G-50 (DNA grade, Pharmacia Biotech, Piscataway, NJ) spin column chromatography. Specific activities of $1.5-3 \times 10^{5}$ cpm/ng were

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obtained. The DNA binding reaction was carried out as described at ambient temperature for 20 minutes (Standke et al., 1994). Nuclear extracts were incubated in a 20 μl reaction containing 10 mM Hepes (pH 7.8), 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 5 mM DTT. Poly (dI-dC) was added in proportion to the protein content of the nuclear extracts (1μg/μg nuclear protein), where same amount of nuclear protein from each sample was added for one run of EMSA. Labeled DNA probe (2μl) was added to the reaction solution at the final step. After completion of the binding reaction, 2μl of loading buffer containing bromphenol blue was added to the solution, and the mixture was loaded immediately on a native 4% polyacrylamide gel. Electrophoresis was run in 0.25x TBE (22mM tris-borate, 0.5 mM EDTA, pH 8.0) at 10V/cm and room temperature with cooling water circulation between the plates. In all the figures except Fig.1, the unbound free DNA probe had run out of the gel so that only Stat5-DNA complex is evident in the auto-radiography. The EMSA gels were used to expose X-ray film, with different times of exposure to ensure that the detected bands were within the saturation limits of the film and then scanned and quantified by Imaging Densitometry (Bio-Rad Laboratories, Hercules, CA). The density volume for each Stat5-DNA complex was deducted from its own lane background. The data are expressed as the density unit of adjusted volume for each treatment.

Stat5 Protein Western Blotting

Mammary nuclear extracts were electrophoresed through 7.5% SDS-polyacrylmide gels run at constant voltage of 100V. Gels were electrotransferred to NitroPure membranes (Micron Separations Inc., Westborough, MA). A Ponceau S

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stain of the membrane was used to confirm equal sample loading. Nonspecific binding was inhibited by incubation of membranes in blocking buffer (10% non-fat dry milk, 0.1% Tween-20 in phosphate-buffered saline) for 1 h at room temperature with shaking, followed by washing in three changes of TBST (0.05% Tween-20) for 5 minutes each. Membranes were incubated with the first antibody (! g/ml) in phosphate buffered saline containing 1% non-fat dry milk and 0.02% Tween-20 for 1 h at room temperature or overnight at 4 °C and washed with TBST three times for 5 minutes each. The primary antibody was rabbit polyclonal anti-mouse Stat5a+b antibody and anti-mouse Stat5a (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-Stat5a+b is shown to recognize both isoforms of Stat5a and Stat5b, and anti-Stat5a is shown to be Stat5a-specific (Gebert et al., 1997). The second antibody (horseradish peroxidase-conjugated anti-rabbit IgG) was incubated with the membranes at 0.1μg/ml for 1 h at room temperature and then washed. ECL detection reagent and Hyperfilm ECL (Amersham, Arlington Heights, IL) was used to detect antibodies bound to the Stat5 protein.

Statistical Analysis

Bands represented Stat5-DNA complex in EMSA and Stat5 immunoblots in Western blotting were analyzed by Imaging Densitometry, and the data are expressed as mean ± SEM of the density unit of adjusted volume (OD*mm*mm). Comparisons between two groups were performed using student t-test and multiple comparisons with ANOVA of SAS (SAS, 1990). Differences with probability less than 0.05 (t-test) were considered significant.

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2.3. RESULTS

Stat5 EMSA and Supershift Assay

We established the Stat5 electrophoretic mobility shift assay using bovine mammary nuclear extract and investigated its DNA binding activity in relation to the amount of nuclear protein added to the binding reaction. Stat5 activity was detected and was proportional to nuclear protein content in the binding reaction. Stat5 activity was almost undetectable at 0.01 μg of nuclear protein, but increased linearly with amount of nuclear protein from 1.0 to 4.0 μg (Fig. 1). When a specific antibody to Stat5 was included in the reaction mixture, the resulting Stat5-DNA-antibody complex (supershifted band) migrated more slowly in the electrophoresis (Fig. 2). This shift in the mobility of the band by a specific antibody confirmed its identity as Stat5. With a decrease in antibody from 2 to 0.1 μg the density of the supershifted band decreased and the density of the band corresponding to the Stat5-DNA complex increased accordingly.

Stat5 Activity in Response to Suckling and Prolactin in Rat Mammary Tissue

Prolactin secretion is strongly influenced by the suckling of pups in rodent (Grosvenor et al., 1979). We investigated Stat5 activity in response to suckling to determine the physiological time course of Stat5 activation by prolactin *in vivo*. It has been shown that mammary Stat5 DNA binding activity in lactating rats is very low at 24 h after removal of their pups, and the activity returns to normal levels at 4 h after restoration of their pups in mice (Schmitt-Ney et al., 1992). We found that rat Stat5 activity responded as early as 30 minutes after restoration of the pups and increased

with time of suckling for 30, 60 and 120 minutes. In a related experiment mammary tissue was obtained from rats 24 h after withdrawl of pups and immediately used to make explants and cultured study the time course of the Stat5 response to PRL *in vitro* (Figure 3). During incubation with PRL, Stat5 activity was detected at 10 minutes and increased linearly for 60 minutes thereafter. These data indicate PRL is able to induce Stat5 activation in rat mammary explant culture to a similar extent and over a similar time course as seen *in vivo*.

In many reported studies of mammary explant culture, PRL is typically added at 1 µg/ml. The physiologic concentration range of PRL in response to suckling in the rat is from 10 to 800 ng/ml (Arbogast and Voogt 1996). We further studied the effects of 200 ng/ml PRL and insulin and hydrocortisone in Stat5 activation (Figure 4). Stat5 DNA binding activity was undetectable in the fresh tissue and in explants incubated with insulin and hydrocortisone alone, but was present in the PRL-supplemented explants. These data indicate PRL at physiologic concetrations is also able to induce Stat5 activation in rat mammary explant culture. Furthermore, the activation of Stat5 is not inducible by insulin and hydrocortisone alone.

Stat5 Response to PRL and GH in Bovine Mammary Explant Culture

Stat5 activity was detected in the fresh tissue collected at 6 h after the last milking (Figure 5). Stat5 activity was also present in explants cultured with insulin and hydrocortisone for 30 minutes but by 60 minutes of incubation, Stat5 activity became undetectable in the presence of only insulin and hydrocortisone. Stat5 activity in the mammary explants was maintained during 60 minutes of incubation in medium

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supplemented with either PRL or GH at 1µg/ml. This experiment was repeated in different lactating cows and showed similar results.

Further experiments were conducted to investigate the dose response of bovine mammary explant culture to PRL and GH (Figure 6). In order to decrease endogenous Stat5 activity in the mammary tissue, tissue samples were taken from lactating cows at 3 days after milking. Stat5 activity was undetectable in the fresh mammary tissue (data not shown) and when the medium was supplemented with only insulin and hydrocortisone. During 1 h-incubation, Stat5 activity in the explants was induced by 50 and 200 ng/ml PRL and 50 ng/ml GH, but not by 5 ng/ml GH. The relative Stat5 activity induced by 200 ng/ml PRL was about twice that induced by 50 ng/ml PRL (P<0.01), and the Stat5 activity induced by 50 ng/ml GH was also significantly higher than that of 50 ng/ml PRL (P<0.05). Stat5 activity was sensitive to PRL and GH at 50 ng/ml, but a low level of GH (5 ng/ml) was unable to independently induce Stat5 activation in the bovine mammary explants.

Combined action of PRL and GH in Stat5 activation

The separate and combined effects of PRL and GH on Stat5 DNA binding activity are shown for rat and bovine mammary explant cultures in Figure 7. The Stat5 activity was undetectable in the rat explants incubated with 50 ng/ml GH, however the response to PRL was higher by 40% in the presence of 50 ng/ml GH (P<0.001). In the bovine mammary explant culture, Stat5 activity was detected in PRL, GH and PRL+GH-treated mammary explants. The relative signal of Stat5 activity was significantly different among the three treatments (P<0.01). Stat5 activity induced by PRL and GH was significantly higher by 40% than by PRL alone. These results

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suggest that Stat5 activation is induced in an additive way by PRL and GH in both rat and bovine mammary tissue.

Stat5 Activation Response to IGF-I in Rat and Bovine Mammary Explants

Experiments were conducted using both rat and bovine explant culture to investigate Stat5 activity response to IGF-I. Prolactin induction of Stat5 activity was used as a positive control, whereas the incubation with only insulin and hydrocortisone was used as negative control (Figure 8A). Stat5 activity was detected in PRL and IGF-I treated explants, but was not induced by insulin and hydrocortisone alone in rat mammary explant culture (Figure 8, A). In bovine mammary explant culture, where Stat5 activity was presented in a low level in the fresh tissue (Figure 8B), PRL and IGF-I supplementation during 30 and 60-minute incubation increased Stat5 activity to a similar degree. This result shows that Stat5 activation responded to IGF-I in both rat and bovine mammary tissues.

Cycloheximide does not influence the response of Stat5 Activity to PRL and GH

It is possible that GH activation of Stat5 might be through increasing mammary IGF-I secretion. The addition of cycloheximide to bovine mammary explant culture at concentrations that block protein synthesis, completely inhibit IGF-I secretion (Campbell et al., 1991). Cycloheximide did not significantly reduce Stat5 activity induced by either PRL or GH (Figure 9), indicating that neither GH nor PRL action on Stat5 activation is via IGF-I production and does not require new protein synthesis.

Stat5 Protein Response to PRL and GH in Rat and Bovine Mammary Explant

Two antibodies to Stat5 are currently commercially available. Anti-Stat5a+b antibody was developed based on the common sequence of both Stat5a and b isoforms, whereas the anti-Stat5a antibody was based on specific Stat5a protein sequence. Anti-Stat5a only reacts with Stat5a, but antibody to Stat5a+b reacts to both isoforms of Stat5. We used both antibodies to detect Stat5 protein in the nuclear extracts from rat and bovine mammary tissue (Figure 10). Stat5 immunoblots showed a signal at the expected molecular weight of around 97 KD (Wakao et al., 1994). A doublet Stat5 band was detected in rat tissue, but two bands were not always clearly resolved in the bovine tissue using anti-Stat5a+b antibody. One clear band was detected in tissue of both species using anti-Stat5a antibody. The upper band obtained using the antibody that reacts to both isoforms corresponds to the molecular size of the single band seen in the Stat5a blots. Thus the upper bands in the rat tissue are Stat5a and the lower bands are Stat5b protein in the anti-Stat5a+b antibody-based Western blotting. Two bands were not always clearly distinguishable in mammary tissue from bovines and this may be due to poorer electrophoretic resolution or possibly with differential specificity of the antibodies towards bovine Stat5 isoforms.

The density of Stat5 protein was linearly related to the amount of nuclear protein loaded on the gel (Figure 11). To determine whether the antibody against both Stat5 isoforms reacted with phosphorylated (active) and dephosphorylated (inactive) forms of the protein, we treated nuclear extracts with protein tyrosine phosphatase (PTP, Figure 12). PTP treatments for 1 to 4 h abolished Stat5 DNA binding activity in EMSA, but did not influence Stat5 protein abundance as determined by Western blot

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Addition of vanadate (an inhibitor for protein phosphatase) to the treatments could maintain the DNA binding activity in the nuclear extracts. These results indicate that the anti-Stat5a+b antibody reacts equally well with the phosphorylated and de-phosphorylated Stat5 protein and thus reflect total Stat5.

We analyzed Stat5 protein in the nuclear extracts previously isolated for determination of Stat5 DNA binding activity. As shown in Figure 13, Stat5 protein abundance, represented by density of Stat5 immunoblots, was not significantly different among the control, PRL, GH and PRL+GH treatments (P>0.05) in the rat mammary explants. Further analysis on bovine mammary nuclear extracts also revealed that PRL and GH treatment during explant culture did not influence Stat5 protein abundance, neither did the cycloheximide treatment of mammary explants (Figure 14, A and B).

67
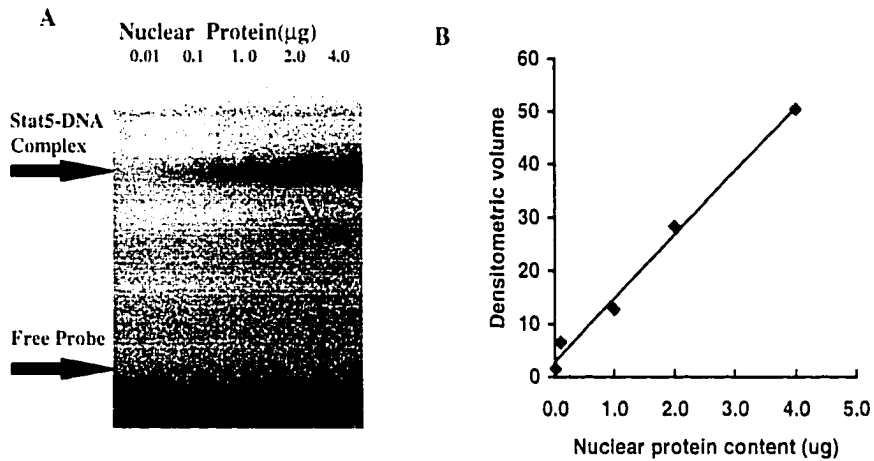
Fig. 2.1. Stat5 activity is proportional to nuclear protein levels. Nuclear

extracts were prepared from mammary tissue of lactating cows. Electrophoretic

mobility shift assay was carried out using the bovine β-casein gene promoter-

based DNA probe and 0.01 to 4 μg nuclear protein. The amount of nuclear

protein in the EMSA is indicated. Stat5-DNA complexes were resolved on 4%

native polyacrymide gel and subject to autoradiography (A). The density of

bands was scanned and quantified using Bio-Rad Molecular Analyst. Stat5 DNA

binding activity is expressed as densitometric volume and plotted against the

content of nuclear protein in the binding reaction (B).

Antibody to Stat5:	0	0	2	1	0.2	0.1
Control Antibody:	0	2	0	0	0	0

Supershifted
band

Stat5-DNA
complex
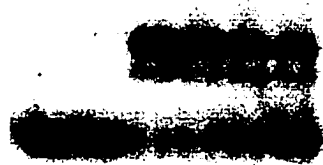
Fig. 2.2. Stat5 EMSA and supershift assay. Nuclear extracts were isolated from mammary gland of a lactating cow. Nuclear protein (2μg for each lane) and varying amounts of antibody to Stat5 from 2 to 0.01 μg were added to the binding reaction with radioactively labeled DNA probe. An antibody to tubulin was used as a control antibody and added to the binding reaction (lane 2). The Stat5-DNA complex and supershift bands were resolved on 4% native separating polyacrylamide with 2% stacking gel and subjected to autoradiography.

69
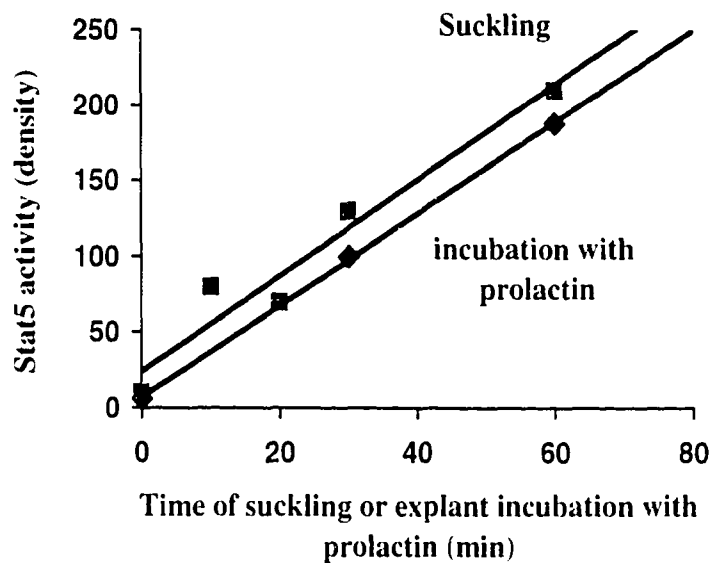
Fig. 2.3. The time course of Stat5 response to suckling and prolactin during explant incubation in rat mammary tissue. Female rats at 10 days of lactation were separated from their pups for 20 h. Pups (7-10) were returned to each female to start suckling for varying times. The female rats were sacrificed and mammary tissue was dissected and quickly frozen until nuclear extraction. For explant incubation with prolactin, mammary tissue was taken from lactating rats, which were separated from their pups for 24 h. Mammary explants were cultured in medium 199 supplemented with 1 μg/ml prolactin, 1μg/ml insulin, 1μg/ml hydrocortisone and 1 mg/ml bovine serum albumin. At the indicated times, the mammary tissues were frozen until nuclear isolation. EMSA was carried out using 4 μg of nuclear protein.

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Prolactin treatment (ng/ml)
Fresh   0   0   200   200

Stat5-DNA
complex

Lane   1   2   3   4   5
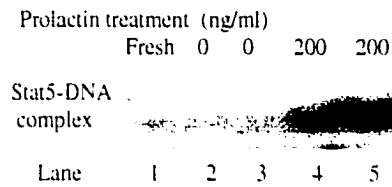
Fig. 2.4. PRL induction of Stat5 DNA binding activty in rat mammary explants. Mammary tissue was taken from female rats at 10 days of lactation. which were separated from their pups for 24 h.   Mammary explants were cultured in medium 199 added with basic culture supplements (1μg/ml insulin, 1μg/ml hydrocortisone and 1 mg/ml bovine serum albumin) and with none (lane 2 and 3) or 200 ng/ml prolactin (lane 4 and 5) for 1h. The mammary tissues were frozen in liquid nitrogen and stored at -70 °C until nuclear isolation. EMSA was carried out using 4 μg of nuclear protein.

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Hormone:	Fresh	BCM	BCM	PRL	GH
Incubation (min)	0	30	60	60	60

Stat5-DNA
complex

| Lane | 1 | 2 | 3 | 4 | 5 |

Fig. 2.5. The response of Stat5 activity to prolactin and growth hormone supplementation in bovine mammary explant culture. Bovine mammary tissue was obtained by biopsy at 6 h after milking. The explants were immediately made and cultured in medium 199, supplemented with 1 μg/ml insulin, 1μg/ml hydrocortisone and 1 mg/ml BSA (basic culture medium, BCM, lane 2 and 3), and the treatment hormones of 1 μg PRL or GH (lane 4 and 5). At the indicated time, mammary tissue was frozen until nuclear isolation. Stat5 EMSA assay was carried out using 4 μg nuclear protein.

72
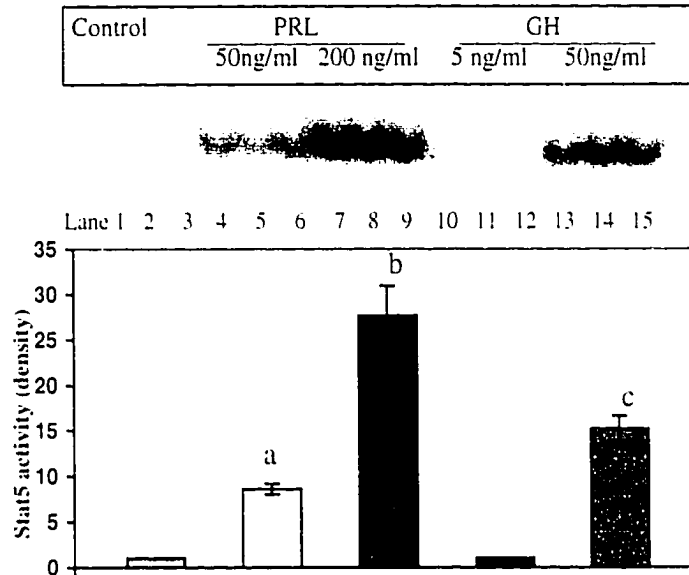
Fig. 2.6. Dose response of bovine mammary explant culture to PRL and GH.

Bovine mammary tissue was obtained from cows that had not been milked for 3 days. The explants were cultured in medium 199, supplemented with $1\mu g/ml$ insulin, $1\mu g/ml$ hydrocortisone and 1 mg/ml BSA (control, lane 1, 2, 3). The treatment hormone PRL (50ng/ml, lane 4, 5, 6; 200ng/ml, lane 7, 8, 9), GH (5ng/ml, lane 10, 11, 12; 50ng/ml, lane 13, 14, 15) were added to the explant culture. The mammary tissue was incubated for 1 h and frozen until nuclear isolation. Stat5 EMSA assay was carried out using 4 μg of nuclear protein. The autoradiographic bands indicated as Stat5-DNA complex were scanned and the mean ± SEM from two experiments with triplicates for each treatment are presented in the graph. Treatments with different alphabetical superscripts are different (P<0.05).

73
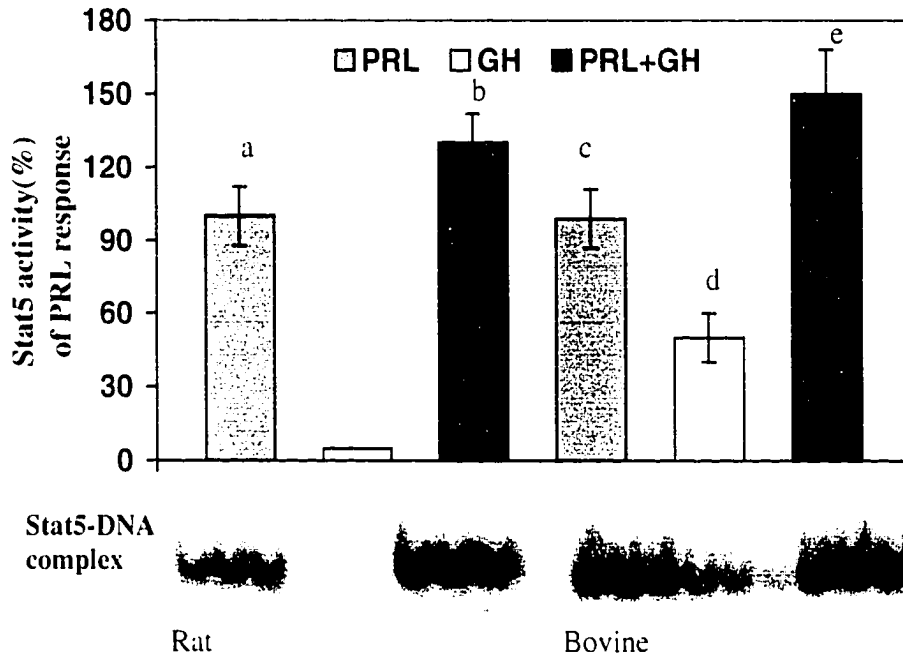
Fig. 2.7. The additive effects of PRL and GH on Stat5 activation in rat and bovine mammary explant. Rat mammary tissue was taken from lactating rats, which were separated from their pups for 20-24 h. Bovine mammary tissue was obtained from lactating cows that had not been milked for 3 days. Mammary explants were cultured in medium 199 supplemented with 1µg/ml insulin and 1µg/ml hydrocortisone and 1mg/ml bovine serum albumin and with treatment hormone PRL (200 ng/ml. three lanes). GH (50 ng/ml. three lanes) and PRL+GH (three lanes). The incubation was carried out for 1h and frozen until nuclear isolation. The Stat5 EMSA assay was carried out using 4 µg of nuclear protein. The rat graph represents the average data of four animals and values are mean ± SEM (n=4). The bovine experiment was run three times with each treatment triplicate in

74
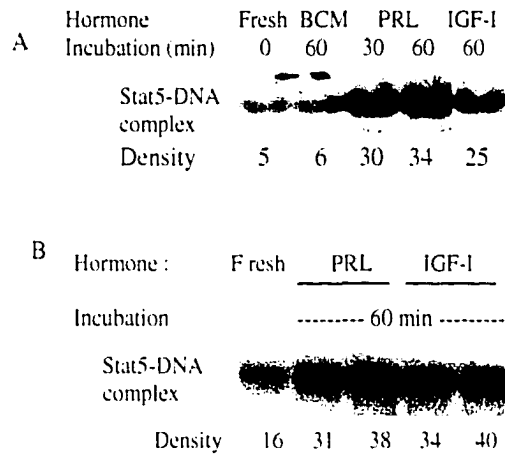
Fig. 2.8. Stat5 activity response to IGF-I in rat and bovine mammary explants. Rat and bovine mammary tissue was obtained from lactating rats that were separated from their pups for 24 h (A) and lactating cows by biopsy at 22 h after milking (B) respectively. Mammary explants were made and cultured for 1h in medium 199, supplemented with 1μg/ml insulin, 1μg/ml hydrocortisone and 1 mg/ml BSA and the treatment hormones of 1 μg /ml PRL or 50 ng/ml IGF-I. At the indicated time, mammary tissue was frozen until nuclear isolation. Stat5 EMSA assay was carried out using 4 μg of nuclear protein.

| Hormone | PRL | | GH | |
| Cycloheximide | None | Present | None | Present |

Stat5-DNA
complex

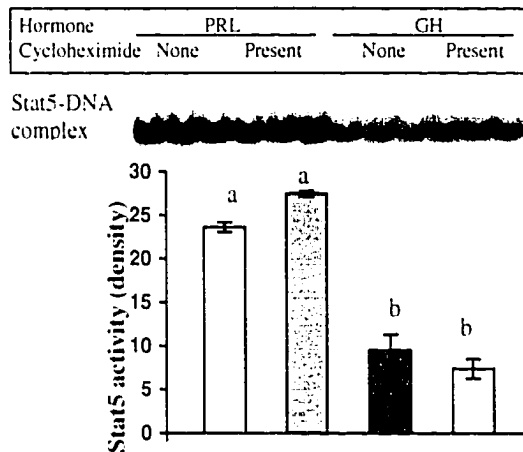**Fig. 2.9. Cycloheximide has no effect on Stat5 activity response to PRL and GH in bovine mammary explant.** Bovine mammary tissue was obtained from lactating cows that had not been milked for 3 days. The explants were cultured in medium 199, supplemented with 1 µg/ml insulin, 1µg/ml hydrocortisone and 1 mg/ml BSA. The treatment hormone 200 ng/ml PRL and 50 ng/ml GH and 0.5 mM cycloheximide were added in the medium as indicated above. Mammary tissue was incubated for 1 h and then frozen until nuclear isolation. Stat5 EMSA assay was carried out using 4 µg nuclear protein. The graph represents the average data (mean ± SEM) from the three animals and only one representative Stat5 EMSA is shown. The significance at different letter is P<0.01 and the same letter is P>0.05.

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Rats   Bovines

Anti-Stat5a+b

Anti-Stat5a
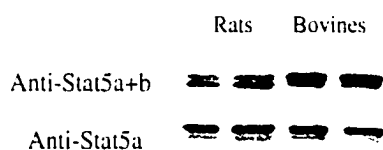
Fig. 2.10. Detection of Stat5 (a and b) Protein in Bovine and Rat Mammary Tissue. Nuclear extracts were isolated from the mammary gland of two lactating rats and two lactating cows. 15 µg of nuclear protein from each sample used for detecting Stat5 protein by Western blotting. The membrane was first incubated with anti-Stat5a+b. Then the membrane was stripped and incubated with anti-Stat5a antibody.
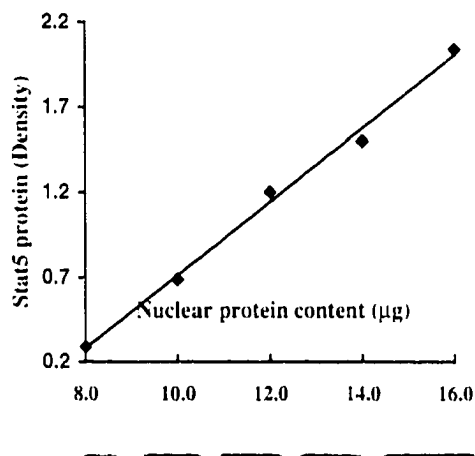
Fig. 2. 11. Detection of Stat5 protein by Western blotting. Nuclear extracts were isolated from mammary gland of lactating cows. Varying amount of nuclear protein (8-16 µg) was separated on 7.5% SDS polyacrylamide gel and eletrophoretically transferred to membrane and immunoblotted with anti-Stat5b antibody. The band of Stat5 (around 97 KD) was quantified and plotted against nuclear protein contents.

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Treatment	Control	PRL	GH	PRL +GH
Stat5 protein				
Density	2.56 ± 0.18	2.93 ± 0.45	2.66 ± 0.03	2.91 ± 0.29

Fig. 2.12. Stat5 protein in response to PRL and GH in rat mammary explants. Female lactating rats were separated from their pups for 20-24 h and sacrificed for obtaining mammary tissue. Mammary explants were made and cultured in medium 199, supplemented with 1μg/ml insulin, 1ug/ml hydrocortisone and 1 mg/ml bovine serum albumin (control). PRL (200 ng/ml) and GH (50 ng/ml) was added to the medium as indicated. Nuclear extracts (15 μg) were used to detect Stat5 protein by Western blotting . The density of the Stat5 band was quantified and mean ± SEM of each treatment is shown.

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A

Hormone	Fresh	BCM		PRL	GH
Incubation (min) 0		30	60	60	60

Stat5 protein

| Density | 3.9 | 5.7 | 6.5 | 5.4 | 4.4 |

B

Hormone	PRL		GH	
Cycloheximide	None	Present	None	Present

Stat5 protein

| Density | 1.77 ± 0.2 | 1.56 ± 0.33 | 1.97 ± 0.05 | 1.96 ± 0.1 |
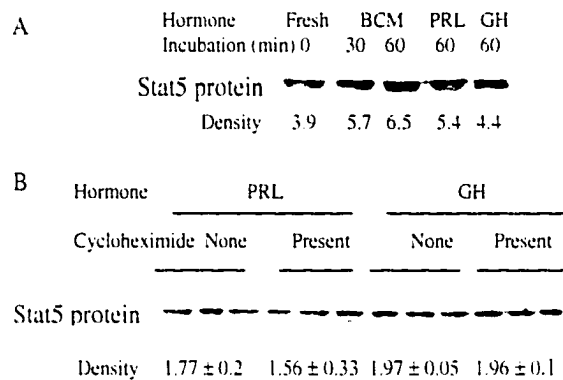
Fig. 2.13. Stat5 protein in response to PRL, GH in bovine mammary explants. Mammary explants, made from lactating cows without milking for 6 h (A) and 3 days (B), were cultured in medium 199, supplemented with 1μg/ml insulin, 1μg/ml hydrocortisone and 1 mg/ml BSA. PRL and GH were added to the medium at the concentration of 200 ng/ml and 50 ng/ml during explant culture. Cycloheximide was added at concentration of 0.5 mM in B. Nuclear extracts (15 μg for A, 10 μg for B) were used to detect Stat5 protein by Western blotting. The density of the Stat5 band was quantified and the individual value or mean ± SEM of each treatment is shown.

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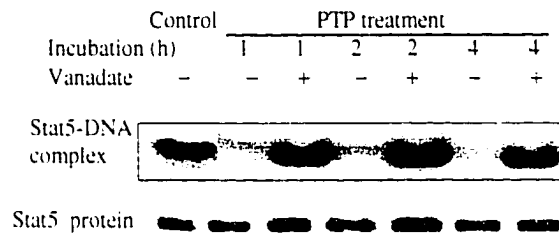
Fig. 2.14. Treatment of nuclear extracts with protein tyrosine phosphatase.
Nuclear extracts were isolated from rat mammary tissue as described except without adding sodium orthovanadate in the extraction buffer. The nuclear extracts were treated with protein tyrosine phosphatase (PTP) for a period of time as indicated. Sodium orthonvanadate (2mM at final concentration) was added to the samples in lanes 3, 5, and 7. Stat5 DNA binding activity was detected with 4 µg nuclear extract by EMSA, and the protein abundance of Stat5 was carried out using 10 µg of nuclear protein by Western blotting.

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2.4. DISCUSSION

Some methodological considerations underlie interpretation of this work. Prolactin, synergistically working with insulin and hydrocortisone, induces casein synthesis in mammary explants (Juergens et al., 1965). Mammary explant appears to be a suitable system to explore the actions of PRL and other hormones on transcription factor Stat5 DNA binding activity and protein. In lactating rats whose pups were removed for about a day, mammary tissue Stat5 activity decreased to an undetectable level. Restoration of suckling rapidly re-induced Stat5 activation and *in vitro* stimulation of mammary tissue with PRL induced Stat5 activity over a similar time scale, suggesting that the incubated tissue was physiologically normal in this response. In freshly isolated bovine mammary tissue from cows milked twice daily, a robust Stat5 DNA binding activity was detected in the electrophoretic mobility shift assay. This apparent Stat5 DNA binding activity was verified by supershift with a specific anti-Stat5 antibody. In the bovine, mammary tissue Stat5 DNA binding activity was reduced at 24 h after cessation of milking and fell to zero at 72 h after the last milking. While it was necessary to stop milking for a relatively long time in the bovine to lower Stat5 activity in the tissue, both bovine and rat explant cultures showed a rapid stimulation of Stat5 DNA binding activity by PRL, GH and IGF-I. They induced Stat5 activity at the high concentrations typically used in explant cultures as well as at levels within physiologic ranges.

Commercially available antibodies were used to detect Stat5 protein. In the rat, two Stat5 isoforms were clearly detected in Western blots using an antibody specific for Stat5a and b and the larger isoform was selectively detected using anti-Stat5a. In the bovine two bands were not clearly resolved in all blots, suggesting

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poorer electrophoretic resolution or differential specificity of the antibodies towards bovine Stat5 isoforms. The experiment with protein tyrosine phosphatase establishes that Stat5 antibodies react equally with phosphorylated and dephosphorylated (inactive) Stat5 and therefore reflect total Stat5 protein in the nuclear extract. This variable was not changed by any of the short-term hormonal treatments used in our study, suggesting that the regulation of Stat5 is predominantly at the level of protein activation. There is only one prior published report of mammary Stat5 in the bovine (Wheeler et al., 1997). In that study bovine tissue showed Stat5 mRNA as well as Stat5 protein in Western blotting. However, either no signal or a very weak signal was detected for Stat5 DNA binding activity in the electrophoretic mobility shift assay. The reasons for the difference in Stat5 activity between these results and the strong activity in our study are unclear, however it seems likely that Stat5 was present but inactive for some reasons in their tissue samples.

Stat5 appears to be a common point in multiple signal transduction pathways in the mammary. In COS cell culture, prolactin, GH, and some cytokines have been independently demonstrated to stimulate the DNA binding activity of Stat5, by co-transfection of vectors encoding Stat5 cDNA and respective hormone receptors (Wakao et al., 1995; Gouilleux et al., 1995). We demonstrated here that Stat5 activity was induced by PRL, GH and IGF-I in mammary explant culture. In the accompanying article (Yang et al., 2000), we show that hormonal infusion and local changes elicited by different frequencies of milking *in vivo* also could influence Stat5 activity in the mammary gland. These results support our hypothesis that Stat5 serves as a common point in the signaling pathways of various extracellular stimuli after they are transduced intracellularly. Though GH and IGF bind their respective receptors and

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activate their specific pathways, they also interact with PRL signaling pathway by activating Stat5. We observed additive effects of PRL and GH action on Stat5 activation in bovine mammary explant culture, and enhanced PRL action in Stat5 activation by GH in rat explants. These data indicate that Stat5 activation could be additive when two stimuli for Stat5 are present simultaneously. Stat5 is also involved in PRL signaling interaction with extracellular matrix in mammary cell culture (Streuli et al., 1995), as well it has functional interactions with glucocorticoid and progesterone receptors in transfected COS cells (Stocklin et al., 1996; Richer et al., 1998). Stat5 may represent part of a common route by which different extracellular signals are transduced in mammary cells. It remains to be determined whether these signals all act on Stat5 through Janus kinase 2, as discussed below.

Both rat and bovine mammary explants showed Stat5 activation at PRL concentrations from within the physiologic levels induced by suckling (10-800 ng/mL in rats and 10-200 ng/mL in the cow, Arbogast and Voogt, 1996; Tucker, 1971). Although the small amounts of tissue available from biopsy precluded the study of a full range of hormone concentrations, Stat5 activity at 50 and 200 ng/mL (Figure 6) suggests a steep dose response and potentially a high degree of sensitivity to PRL concentrations. We thus speculate that Stat5 activity could be regulated over a broad range of activation by PRL alone in both species. Growth hormone is more likely to modulate the PRL response. In rat mammary explants GH only induced Stat5 activity at a pharmacological concentration of 1 μg/ml, and GH at 50 ng/ml (about 2-5 fold the physiologic concentration) was ineffective by itself in stimulating Stat5 activity. However, in the presence of 50 ng/ml GH (an otherwise ineffective dose), the response of Stat5 to 200 ng/mL PRL was increased in the rat compared with PRL

addition alone. Since GH concentrations do not change in response to suckling or milking (Escalada et al., 1997; Tucker, 1971), it may be that it acts as a secondary factor modulating the PRL response or setting a basal level of activation. This type of relationship has been demonstrated in the human interleukin-3-dependent erythroleukemia cell line F-36P, where IGF-I augmented erythropoeitin-induced Stat5 activity but alone failed to induce Stat5 activation (Okajima et al. 1998). Growth hormone at 50ng /ml, which was ineffective in the rat, induced Stat5 activity by itself in bovine mammary explant culture and this response was slightly greater than that seen with 50 ng/ml PRL. This may reflect a lower threshold for activation of Stat5 by GH in the bovine. Although this concentration is above the physiologic range, it is a level achieved in GH infusion trials in lactating cows, and it may explain why GH infusion increases milk synthesis in cattle but not in laboratory rats (Etherton and Bauman 1998; Flint, 1995).

Bovine and rat mammary tissues in explant culture have the capacity to respond to GH through a signal transduction cascade involving Stat5. Direct and specific induction of Stat5 activity by GH has been shown in COS cells transfected with the GH receptor (Argetsinger et al. 1993). However, GH action in mammary is thought to be effected by a direct action of GH upon its receptor and also by an indirect action mediated by IGF-I. There is evidence that both GH and IGF-I receptors are present in bovine mammary cells (Glimm et al. 1990, Rechler, 1985). GH-induced activation of Stat5 was rapid and was not inhibited by cycloheximide, an agent that inhibits the production of IGF-I in mammary cells (Campbell et al 1991). It seems likely that there is a GH-receptor mediated action on Stat5 activity and an additional effect of IGF-I *per se*. Here we present the first observation that IGF-I

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stimulation is associated with activation of Stat5. This response must entail activation of a kinase. There is an IGF-I receptor-associated kinase and a number of kinases are known to be involved in IGF-I signal transduction, including insulin receptor substrate-I and phosphatidylinositol-3-kinase (Jones and Clemmons, 1995; Melmed et al., 1996). It remains to be established whether IGF-I activated Stat5 via Janus kinase 2 or one of these other enzymes.

This work is limited in its overall interpretability because of the lack of concurrent measures of β-casein transcriptional activity, which was precluded by sample availability. We did not provide experimental data to clarify if the increased Stat5 activity results in increased gene transcription activity during explant culture. No evidence has been obtained here to suggest that either GH or IGF-I result in activation of casein gene transcription during explant culture. In COS cells transfected with GH receptor, Stat5 DNA binding was activated by GH addition, however, there was no association with transcriptional activity of the reporter gene that was linked with β-casein gene promoter. The COS cell transfection with PRL receptor shows PRL stimulation of the reporter gene transcriptional activity (Gouilleux et al., 1995). In Stat5a-deficient transgenic mice, while full lobuloaveolar development was not observed, milk protein gene was expressed in the tissue, suggesting that Stat5 is not mandatory for milk protein gene expression (Hennighausen et al., 1998). It is known that Stat5 is involved in regulation of cell development such as observations of Stat5 during mammary development and its influence in liver function (Liu, et al. 1995; Udy et al., 1997). Regulatory protein p21[WAF1] and c-fos genes in cell development contain one or more Stat5-binding sites and their transcriptional activation responded to Stat5 induction (Richer et al., 1998). Therefore, transcription factor Stat5 may not

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only regulate milk protein genes but also other genes related to cell development in the mammary gland. The Stat5 pathways play an important role in transducing lactogenic hormone signals in the mammary cell, however, the contribution to the total milk protein mRNA pool made from increased Stat5 activity by PRL, GH and IGF-I has not been established.

In conclusion, our work provides evidence that PRL and GH independently induce Stat5 activation in bovine mammary explant culture. In both rat and bovine explants, PRL and GH showed additive effects in Stat5 activation. We also demonstrate that IGF-I signal transduction pathway may involve Stat5 in rat and bovine mammary cells. These results from in vitro explant culture indicate that transcription factor Stat5 serves as a common point of signal transduction pathways of several lactogenic hormones in the mammary gland.

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CHAPTER 3. PHYSIOLOGICAL VARIATIONS OF STAT5 IN BOVINE MAMMARY GLAND

3.1. INTRODUCTION

Milk synthesis is controlled by a multitude of physiological influences with hormones playing essential roles in both initiation and maintenance of lactation. In lactating cows, growth hormone (GH) is known to be one of the predominant circulating galactopoietic hormones, and infusion of GH elevates levels of milk synthesis and milk yield (Bauman and Vernon 1993). Milk synthesis in the mammary gland is also under a degree of hormone-independent local control. For example, studies in which the mammary quarters were milked separately at different daily frequencies demonstrate a positive relationship between milk yield and frequency of milking (Linzell and Peaker, 1971). The action of GH on lactation is proposed to be mediated via insulin-like growth factor I (IGF-I, Etherton and Bauman, 1998), as well as by direct effects via a specific GH receptor in the mammary gland (Glimm et al., 1990). The mechanisms of the local control with varying frequency of milking are thought to involve feedback inhibition of protein synthesis by a milk protein (Wilde et al., 1995). Thus the regulatory pathways originating from increased GH circulation in stimulating milk synthesis are quite different from those elicited by different milking frequency. However, both of these stimuli are transduced and act ultimately on milk protein gene expression to enhance milk production. It is not known how these physiological stimuli coordinately regulate milk synthesis *in vivo*, especially the molecular connection of their respective signaling pathways in the mammary gland.

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Recently, transcription factor Stat5 (signal transducer and activator of transcription 5) has been suggested to play a key role in regution of milk protein gene expression. Stat5 mutation results in loss of β-casein promoter activity and β-casein transcription in transfected COS cells (Schmitt-Ney et al., 1991, Standke et al., 1994). Two forms of Stat5 (a and b) were identified in mice and Stat5a-deficient transgenic mice failed to lactate, suggesting its mandatory role in lactogenesis (Liu et al. 1995 and 1997). In cultured cells, Stat5 is activated by several hormones, including growth hormone (GH) and prolactin GH (Gouilleux et al., 1995, Ruff-Jamison et al., 1995, Wakao et al., 1995).

We have recently been interested in establishing the role of Stat5 in regulation of lactation in the bovine. In a related study, we hypothesized that Stat5 may serve as a common point in the signal transduction pathways of various lactogenic hormones in bovine mammary cells, which are regulated by GH and insulin-like growth factor (IGF-I) in addition to PRL. We established and validated assays of Stat5 activity and protein abundance in the bovine. Bovine explant culture showed a rapid stimulation of Stat5 DNA binding activity by PRL, GH and IGF-I, at the high concentrations typically used in explant cultures as well as at levels within physiologic ranges. These results suggest that transcription factor Stat5 may represent part of a common route by which different extracellular signals converge and are transduced intracellularly to coordinately regulate cell function in the mammary gland.

In the present study we sought to extend our prior observations obtained *in vitro* with studies of the physiologic regulation of Stat5 DNA binding activity and protein level *in vivo*. Thus we hypothesized that signal transduction cascades generated from

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physiological stimuli of milk synthesis such as increased levels of circulating GH and

local stimuli associated with milking frequency would converge upon Stat5, and

influence mammary Stat5 activation *in vivo*.

3.2. MATERIALS AND METHODS

Animals.

Studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care. For studies of Stat5 activity in relation to stage of lactation, 12 Holstein cows from the Dairy Research and Technology Centre of the University of Alberta were used. Cows were fed a total mixed ration containing 50% concentrate and 50% forage and housed in tie-stalls with 24 h light, and milked twice per day at 4 AM and 4 PM. Mammary tissue was obtained by surgical biopsy 2 h before PM milking (Knight et al., 1992). Two non-lactating cows in late gestation (230-245 days) were also biopsied. Mammary tissue samples from 18 Holstein cows provided by Dr. H. A. Tucker (Michigan State University, East lansing, MI), were used to study Stat5 activity in relation to GH and growth hormone-releasing factor (GHRF) treatments. These cows were in mid-lactation, and received one of three treatments (1) continuous i.v. infusion of 29 mg/d recombinant bovine GH (Somavubove, Pharmacia & Upjohn Co. Peapack, NJ), (2) continuous i.v. infusion of 12 mg/d recombinant bovine GHRF (Somavubove, Pharmacia & Upjohn Co. Peapack, NJ), and (3) no infusion (controls) for 63 days. Cows were killed at about 8 h after PM milking and mammary tissue was obtained within 20 minutes after killing (Binelli et al., 1995).

Five cows in mid-lactation were used to study mammary Stat5 in response to milking frequency, which was implemented by milking left and right quarters of the mammary gland separately in each cow. Right quarters of the mammary gland of each cow were milked twice per day at 4 AM and 4 PM, and the left quarters were milked once daily at 4 PM for two weeks. Milk yield was recorded at each milking and milk

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protein concentration was determined by infra-red analytic instrument (Robinson and

Kennelly, 1989). At the end of the 18 day milking schedule, mammary tissue from

front right and left quarters was separately biopsied 2 h just before the 4 PM milking.

Mammary tissue obtained by biopsy and from slaughtered animals was immediately

placed in liquid nitrogen and stored at -80°C until nuclear extraction.

Preparation of Nuclear Extract

All the chemicals used in the following analysis were purchased from Sigma

(St Louise, MO) unless otherwise stated. Mammary nuclear extracts were prepared as

previously described (Standke et al., 1994) with some modifications (Yang et al.,

2000).

Electrophoretic Mobility Shift Assay (EMSA)

The procedure for this assay was previously established (Standke et al., 1994).

The Stat5 binding site (5'-AGATTTCTAGGAATTCAAATC-3') based on the bovine

β-casein promoter was used to design the probe for EMSA. We have previously

described EMSA for Stat5, and shown this assay to be specific in detection of bovine

mammary Stat5 by "supershift" of the observed band for Stat5-DNA complex with an

anti-Stat5 antibody. The assay is linear with protein content of the nuclear extracts

(Yang et al., 2000). Briefly, the doubled stranded labeled DNA probe was obtained by

fill-in reaction with [α-^{32}P]dATP. The DNA binding reaction (20 μl) was carried out

as described (Yang et al., 2000) at room temperature for 20 minutes. Poly (dI-dC) was

added in proportion to the protein content of the nuclear extracts (1 μg/μg nuclear

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protein). Labeled DNA probe (2 µl) was added to the reaction solution. The Stat5-DNA complex was separated on a native 4% polyacrylamide gel. The gel was dried and used to expose an X-ray film.

Stat5 protein Western Blotting

Nuclear protein electrophoresis and Western blotting were run as described (Wakao et al., 1995) with minor modifications (Yang et al., 2000). Nuclear protein was run on 7.5 % SDS-polyacrylamide gels. Gels were electrotransferred to NitroPure membranes (Micron Separations Inc., Westborough, MA). Nonspecific binding was inhibited by incubation of membranes in phosphate buffered saline (PBS) with 10% non-fat dry milk and 0.1% Tween-20 for 1 h at room temperature. Membranes were incubated with 1 µg/ml anti-Stat5 antibody in PBS with 1% non-fat dry milk and 0.02% Tween-20 for 1 h. An antibody from Santa Cruz Biotech (Cat. # sc-835, Santa Cruz, CA), which recognizes both Stat5a and Stat5b, was used in all Western blots. We have previously shown that the Western blotting detects both tyrosine-phosphorylated (active) and dephosphorylated (inactive) Stat5 and thus represents total Stat5 protein. The assay is linear with protein amount of nuclear extract (Yang, et al., 2000).

Statistical Analysis

The bands on EMSA and Western blots were analyzed by Imaging Densitometry (Bio-Rad Laboratories) and data are expressed in units of adjusted volume (optical density * mm *mm). The least square means and standard errors of the means were obtained from the General Linear Model (GLM) of SAS (SAS, 1990).

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Significant differences between treatment or group means were taken from the matrix

of student t- test within each set of least square means. Significance was determined at

ˉ P<0.05.

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3.3. RESULTS

Stat5 DNA Binding Activity and Protein Abundance in Bovine Mammary Gland

Stat5 DNA binding activity was detected in the mammary gland of lactating cows, but no Stat5 activity was observed in non-lactating, non-pregnant cows (Figure 1). We investigated Stat5 activity in mammary tissue samples from 9 cows at various stages of lactation (Figure 2). Three cows in early lactation (30 to 53 days) had a similar mammary Stat5 activity and protein abundance. However, mammary Stat5 activity showed considerable variations in the five cows between 90-110 days of lactation. One cow in late lactation (218 days in lactation) had similar Stat5 activity to cows in earlier stages of lactation. The same nuclear extracts were further used to detect Stat5 protein by Western blotting. The density of the bands varied among cows at similar stages of lactation, but showed less variably than that of Stat5 DNA binding activity. These results demonstrate the presence of Stat5 in the mammary gland throughout lactation; however, the variability in mammary Stat5 and small sample size precluded any determination of any relation to lactational stage.

Stat5 DNA Binding Activity and Protein Abundance in Mammary Gland of Control, GH and GHRF Treated Cows

One potential source of variations of *in vivo* Stat5 protein and activity may be from the prevailing hormonal levels of individual animals. Mammary Stat5 was analyzed in GH and GHRF treated animals, in which chronic infusion of GH and GHRF significantly increased circulating blood GH levels and milk yield (Dahl et al.,

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1993). Mammary Stat5 activity was significantly lower in GH (-50%) and GHRF (-60%) treated cows than that in control cows (n=5; P<0.05, Figure 3). GH and GHRF treatment had a tendancy to suppress Stat5 protein abundance (P<0.1) compared to control cows, however this effect was too small to account for the reduction of Stat5 activity (Figure 4).

Mammary Stat5 Response to Different Levels of Milking Frequency

Five lactating cows were used to study the influence of once *versus* twice daily milking on mammary Stat5 activity. Left and right quarters of the mammary gland were separately milked once and twice daily respectively for 18 days. Milk and milk protein yield were influenced by the milking schedule (Table 1). Once daily milking significantly reduced milk yield and milk protein yield in comparison with twice daily milking for days 1 to 3 and days 16 to 18. Milk protein concentration for 1X milking appeared to increase during day 1 to 3 (P<0.01), but it was not significantly different between 1X and 2X milking on day 16 to 18 (P>0.05).

In the EMSA, Stat5 DNA binding activity was significantly depressed (-19%) by once daily milking (left quarters) in comparison with twice daily milking (right quarters) (P<0.01, Figure 5). Analysis of Stat5 protein abundance in the nuclear extract showed that left quarters (1X) had lower level of Stat5 protein (-36%) than the right quarters (2X) (P<0.01, Figure 6). The fall in Stat5 protein in the quarters milked only once per day was large enough to account entirely for the reduction in Stat5 activity.

Stat5 Activity in Relation to Average Milk Protein Concentration

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Since Stat5 is a transcription factor involved in regulation of milk protein gene expression, and levels of milk protein production show considerable variability amon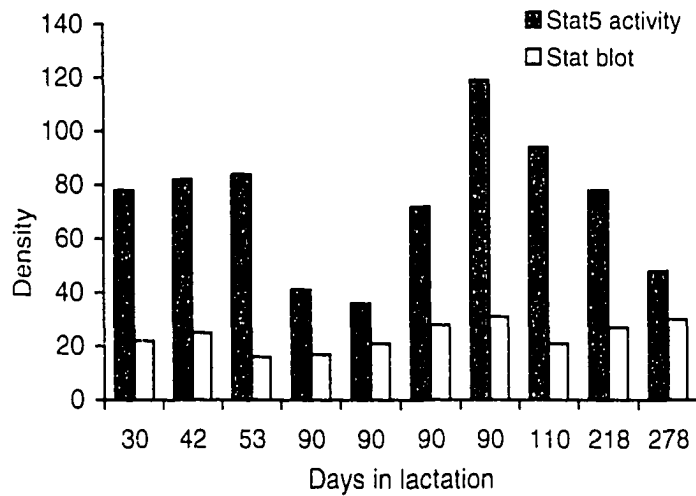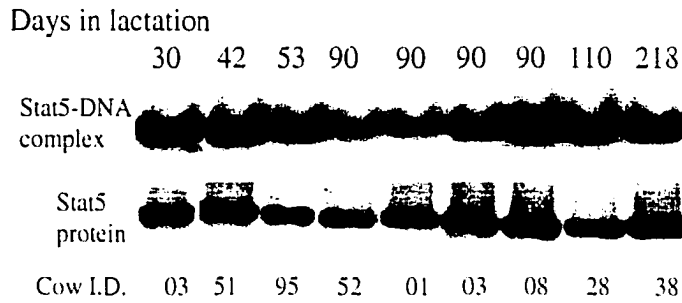g cows at similar lactational stages, the physiological variability in mammary Stat5 may represent differences in milk protein synthesis of cows. We analyzed Stat5 activity in relation to milk protein concentration (Figure 7). Within each treatment (control, GH, GHRF), animals were classified into either a low or a high average milk protein concentration. When control and GHRF-treated cows were divided into groups based on their milk protein concentration, there was significantly higher Stat5 activity in the mammary tissue of animals producing higher protein concentrations (Figure 7A,B). In this experiment the GH treated cows showed a relatively small range of milk protein concentrations (Figure 7C), and there was no relation with Stat 5 activity in this group. To evaluate the overall relationship of mammary Stat5 and milk protein concentration, we carried out regression analysis on the pooled data of control, GH and GHRF treated cows based on their relative Stat5 activity. This analysis revealed that mammary Stat5 was correlated to average milk protein concentration ($n=18$, $r=0.505$, $P<0.05$, Figure 8), but not significantly related to milk protein yield (data not shown, $P>0.05$). Using anti-Stat5 antibody, the protein abundance of Stat5 was analyzed in the same tissue samples, there were no significant differences between animals producing high and low protein concentrations in control and GHRF treated cows (Figure 9). The nuclear extracts of the mammary tissue from GH treated cows were not available for Stat5 protein analysis.

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Lactating non-lactating

Stat5-DNA
complex

Free Probe

Lane 1 2 3 4
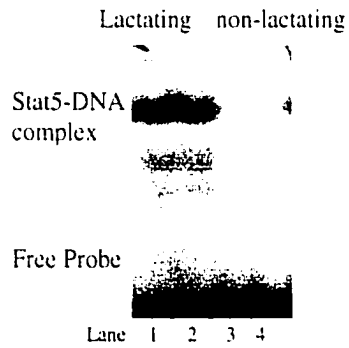
Fig. 3.1. Stat5 activity was detected in mammary gland from lactating cows, but not from non-lactating and non-pregnant cows.

Nuclear extracts were prepared from mammary tissue samples of two lactating cows (lane 1 and 2) and two non-lactating and non-pregnant cows (lane 3 and 4). EMSA was performed using 4 µg of nuclear protein and radioactively labeled DNA probe.

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Days in lactation

| 30 | 42 | 53 | 90 | 90 | 90 | 90 | 110 | 213 |

Stat5-DNA complex

Stat5 protein

Cow I.D. 03 51 95 52 01 03 08 28 38

Fig. 3.2. Stat5 activity and protein in the mammary gland of cows at different stages of lactation

Nuclear extracts were prepared from ten mammary tissue samples of lactating cows. Days in lactation for each cow were shown above each lane. EMSA was performed by 4 μg of nuclear proteins from each sample, and 15 μg of nuclear protein was used for Western blotting for detecting Stat5 protein. The density of the band for Stat5 activity and protein was scanned and charted in the graph.

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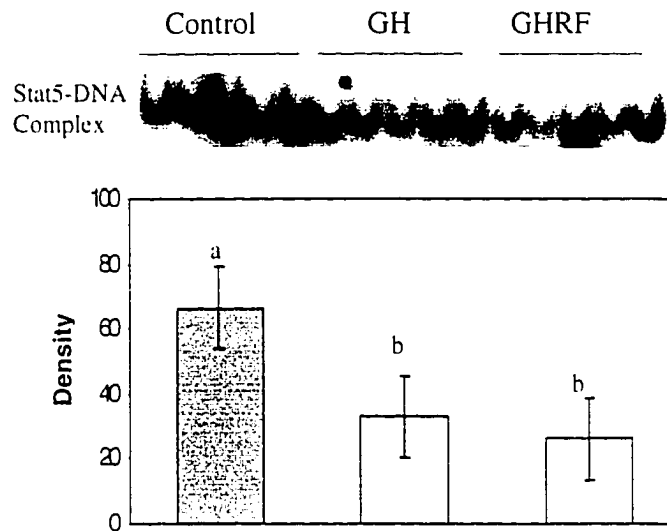
Fig. 3.3. Stat5 activity in the mammary gland of GH and GHRF-treated cows.

The mammary tissue was obtained from 15 cows, in which 5 cows were from control, 5 cows received GH treatment and 5 cows received GHRF treatment for 63 days. Nuclear extracts were isolated from the mammary tissue and 4 µg of nuclear protein from each cow were used in the Stat5 binding reaction with the β-casein promoter-based DNA probe. Stat5-DNA complex for each sample was obtained by EMSA. The density of the Stat5-DNA complex bands were scanned and quantified. The least square means and standard error of the densities from each treatment (n=5) are shown in the graphics. Bars identified by different letter differ at P<0.05.

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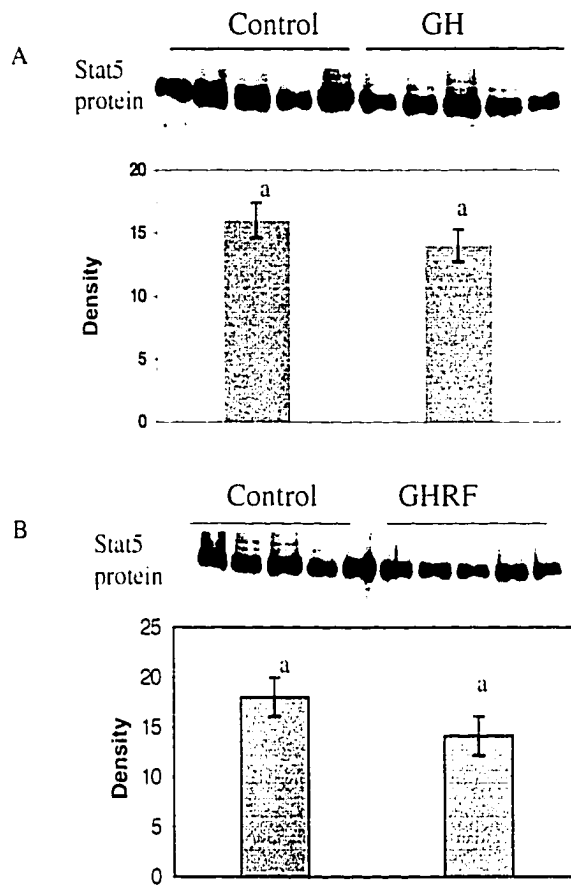
Fig. 3.4. Stat5 protein abundance in the mammary gland of GH and GHRF treated cows.

The mammary tissue was obtained from 15 cows, in which 5 cows were used as controls, 5 cows received GH treatment and 5 cows received GHRF treatment for 63 days. Nuclear extracts were isolated from the mammary tissue and 15 μg of nuclear protein from each cow were resolved on 7.5 % SDS polyacrylamide gels. Two separate gels were run for control vs GH treatment (A) and control vs GHRF treatment (B), and Stat5 protein was detected by Western blotting. The density of the bands were scanned and quantified. The least square means and standard error of the densities from each treatment (n=5) are shown in the graphics. Bars identified by different letter differ at P<0.05.
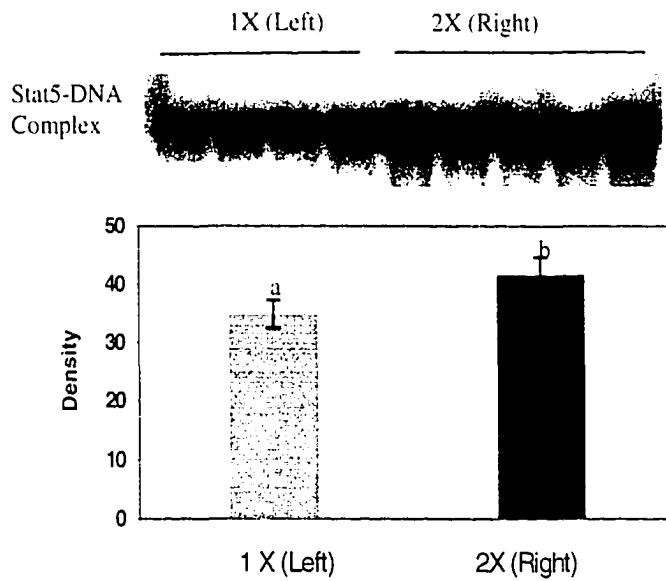
Fig. 3.5. Mammary Stat5 activity in response to once and twice daily milking

Five cows in mid-lactation were milked daily once on the left quarters and twice on the right quarters of the mammary glands for 18 days. Mammary tissue was surgically biopsied at the end of the milking schedule from front left and right quarters respectively. Nuclear extracts were isolated from the mammary tissue and 4 μg of nuclear protein from each cow was used in the Stat5 DNA binding reaction and Stat5-DNA complex was determined by EMSA. One nuclear extract sample from the left quarter of a cow was not available for Stat5 analysis. The density of the bands were scanned and quantified. The least square means are shown in the graphics. Bars identified by different letter differ at P<0.01.

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IX (Left)   2X (Right)
_____   _____
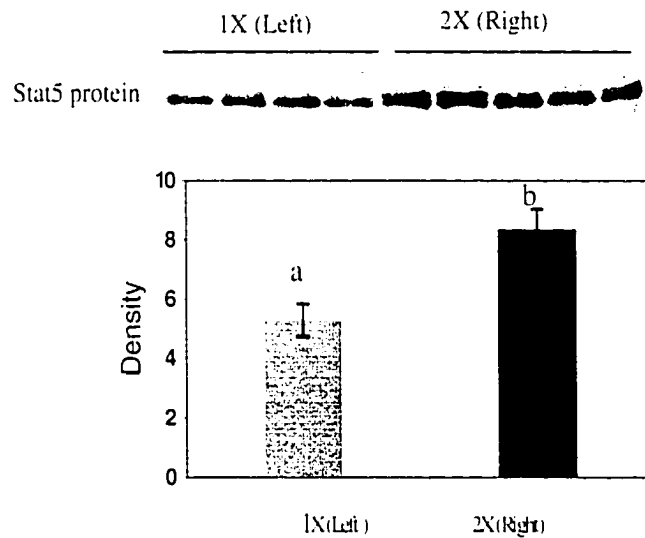
Stat5 protein   ▬▬ ▬▬ ▬▬▬▬ ▬▬▬ ▬▬ ▬▬ ▬▬ ▬▬

Fig. 3.6. Mammary Stat5 protein abundance in response to once and twice daily milking

Five cows in mid-lactation were milked daily once on the left quarters and twice on the right quarters of the mammary glands for 18 days. Mammary tissue was surgically biopsied at the end of milking schedule from front left and right quarters respectively. Nuclear extracts were isolated from the mammary tissue and 20 µg of nuclear protein from each sample were used in Western blotting system to determine mammary Stat5 protein abundance. One nuclear extract sample from the left quarter of a cow was not available for Stat5 analysis. The density of the bands were scanned and the least square means and standard error were shown below the graphic picture. Bars identified by different letter differ at $P < 0.01$.

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A

Milk protein concentration (%)

3.04 ± 0.007   3.22 ± 0.030

Stat5-DNA
complex

Density   37.1 ± 4.5   53.1 ± 3.2   *

B

Milk protein concentration (%)

3.00 ± 0.051   3.24 ± 0.074

Stat5-DNA
complex

Density   32.1 ± 8.0   54.9 ± 9.5   *

Milk protein concentration (%)

2.97 ± 0.046   3.17 ± 0.078

C   Stat5-DNA
complex

Density .   70.4 ± 10.6   71.2 ± 15.4   n.s.
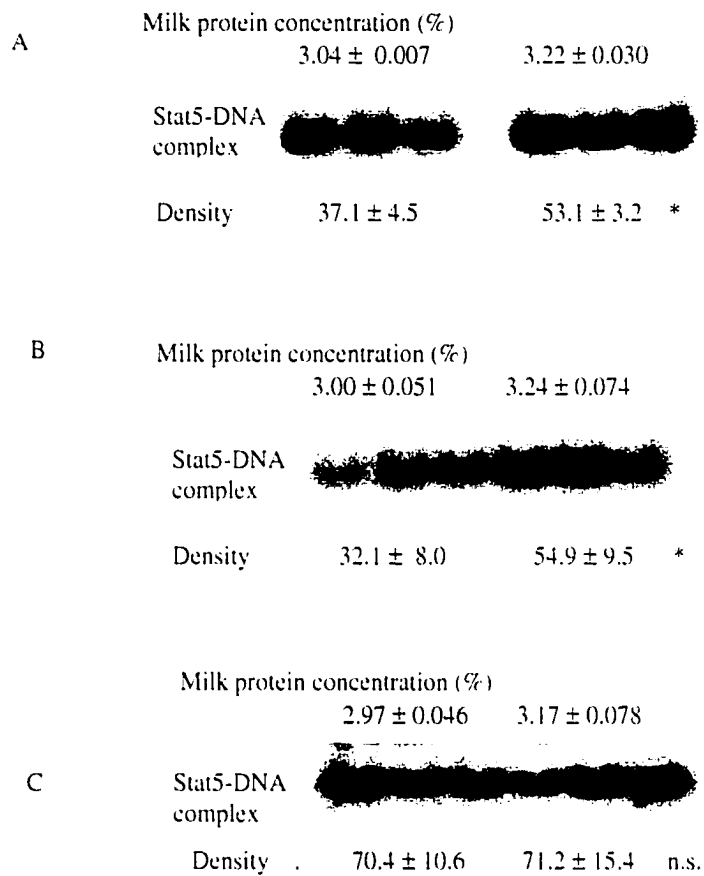
Fig. 3.7. Mammary Stat5 activity in relation to milk protein concentration in control, GH and GHRF treated cows

Nuclear extracts were isolated from the mammary gland of 18 cows with 6 cows for each treatment. The Stat5 activity was analyzed in two groups of cows with high and low average milk protein concentration within each treatment. Fig.7A, B and C showed Stat5 activity for control, GHRF and GH treated cows respectively. The Stat5-DNA binding reaction was carried out by 4 µg from each sample and Stat5-DNA complex was detected by EMSA. The density of each band was scanned and quantified. The least-square means and standard error were shown. *: $P<0.05$ and ns: not significant.
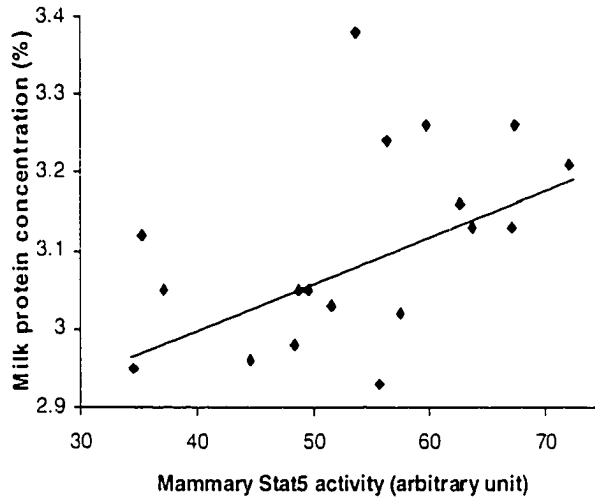
Fig. 3.8. Mammary Stat5 activity in relation to milk protein concentration.

Mammary Stat5 was detected by EMSA and the density of Stat5-DNA complex from control, GH and GHRF treated animals were determined. The milk protein concentration was plotted against mammary Stat5 activity in the pooled data.

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A

Milk protein concentration (%)

3.04 ± 0.007   3.22 ± 0.030

Stat protein

Density   .   10 ± 1.7   11 ± 2.2   ns

B

Milk protein concentration (%)

3.00 ± 0.051   3.24 ± 0.074

Stat5 protein .

Density .   6.1 ± 0.6   5.3 ± 1.0   n.s.
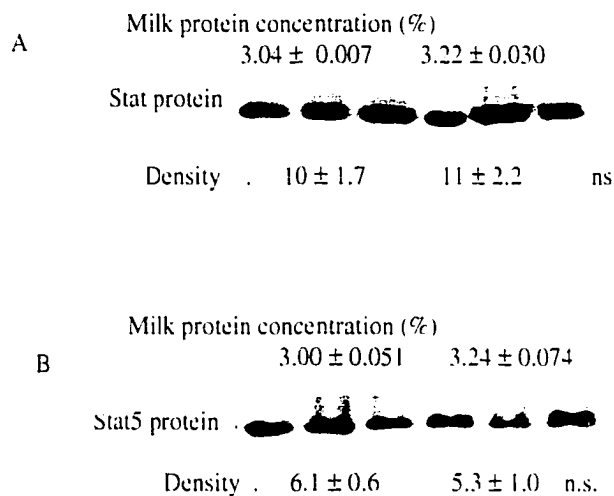
Fig. 3.9. Stat5 protein abundance in relation to milk protein concentration.

The mammary tissue was obtained from 12 cows. Nuclear extracts were isolated from the mammary tissue, and Stat5 protein abundance was determined by Western blotting, where 20 μg of nuclear protein from each sample were used. The density of the bands were scanned and quantified and the least square means and standard error were shown below the graphic picture. A and B represented the mammary tissue samples from control and GHRF treated cows respectively.

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Table 1. Influence of milking frequency on milk and milk protein yield*

Parameters	1X (left quarters)	2X (right quarters)	Significance
Day 1 to 3			
Milk yield (kg/d)	8.35 ± 0.46	14.26 ± 1.92	P<0.05
Milk Protein (%).	3.22 ± 0.03	3.08 ± 0.04	P<0.01
Milk protein yield (kg/d)	0.27 ± 0.02	0.44 ± 0.06	P<0.05
Day 16 to 18			
Milk Yield	9.15 ± 0.84	16.6 ± 1.21	P<0.01
Milk protein (%)	3.29 ± 0.05	3.26 ± 0.02	P>0.05
Milk protein yield (kg/d)	0.30 ± 0.03	0.55 ± 0.04	P<0.05
Day 1 to 18			
Milk yield (kg/d)	8.6 ± 0.75	15.5 ± 1.42	P<0.001

* n=5

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3.4. DISCUSSION

Sources of variation of Stat5 activity *in vivo*

The general pattern of Stat5 activity in bovine mammary gland was similar to that reported in rodents. Stat5 activity was expressed just prior to and throughout lactation in the mammary gland but was absent in dry, non-pregnant cows. In mice, Stat5 activity was detected at day 15 of pregnancy, remained strong during lactation and ceased at the end of lactation (Schmitt-Ney et al., 1992). There is only one prior published report of mammary Stat5 in the bovine (Wheeler et al., 1997). In that study bovine tissue showed Stat5 mRNA as well as Stat5 protein in Western blotting. However, either no signal or a very weak signal was detected for Stat5 DNA binding activity in the electrophoretic mobility shift assay. The reasons for the difference in Stat5 activity between the results of Wheeler et al. (1997) and the strong activity in our study are unclear, however it seems likely that Stat5 was present but inactive for some reason in their tissue samples.

In our studies, all tested lactating animals showed a robust Stat5 DNA binding activity. There substantial inter-individual variations in this activity among animals at similar stages of lactation. This variation has implications for the study of Stat5. In physiologic experimentation on dairy cattle mammary tissue is available from animals killed at the end of study which is costly, or from biopsy which is both costly and invasive. These factors tend to limit sample size in studies of dairy cattle and it is thus important to identify and control for sources of variation. Diurnal variation in milking stimulation would appear to be one source. In the accompanying paper we obtained evidence for a progressive decline in Stat5 activity over time since the last milking

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(Yang et al., 2000). When mammary tissue was sampled at a constant interval after the last milking (i.e. Fig. 5) and controlled comparison made within animals significant differences of the order of 20% in Stat5 activity could be detected with a sample size of 5.

Hormonal status of the animals is likely a contributor to level of Stat5 activity within any individual animal. Stat5 DNA binding activity in bovine mammary explants appears highly sensitive to prolactin concentrations within the physiologic range and we suggest that Stat5 could be regulated *in vivo* over a broad range of activation by PRL alone (Yang et al. 2000). In explants, GH alone at a physiologic concentration of 5 μg/ml did not by itself stimulate Stat5 activity. At 50 μg/ml, a concentration achieved in some infusion trials, GH stimulated Stat5 activity and potentiated the response to prolactin. Our results obtained *in vivo* support the idea that hormonal status of individual animals will be an important source of variation in Stat5 activity. The infusion of GH and GHRF for two months significantly increased circulating GH levels and in these cows overall milk yield at the end of the study (when animals were sacrificed for tissue sampling), was significantly increased by GH and GHRF treatment (Binelli et al., 1995). Stat5 activity was reduced in the mammary gland by GH and GHRF infusion. Additional studies are required to determine whether this change might have resulted from GH- induced alterations in prolactin levels (Borromeo et al., 1995; Cecim et al., 1995) or from a down – regulation in the overall train of signal transduction in response to elevated GH levels.

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Stat5 DNA Binding Activity and Protein Response to Milking Frequency

In the bovine the four quarters of mammary gland are anatomically separated. Thus by altering milking frequency in different quarters, we were able to study local effects in a model where all four quarters could be presumed to receive the same hormonal stimuli through the common blood supply. The DNA binding activity and protein level of Stat5 in the mammary gland of lactating cows was depressed by once daily milking in comparison with twice daily milking. The magnitude of the decrease in Stat5 protein was greater than th decrease in Stat5 activity. The change of Stat5 protein thus can account entirely for the alteration in Stat5 activity.

Modulation of Stat5 in response to milking frequency adds support to the idea that Stat5 serves as a common point of the signaling pathways of different physiologic stimuli *in vivo*. Stat5 may be not only involved in signaling pathways of multiple hormones, but also convey signals intracellularly from non- hormonal stimuli. It is not known what specific signals could influence Stat5 protein and activation in response to milking frequency. We speculate that there may be two regulatory signals that are candidates for local activation of Stat5. Firstly, it has been reported that milk synthesis is regulated by autocrine control through a milk protein, known feedback inhibitor of lactation (FIL) and this protein inhibits milk synthesis (Wilde et al., 1995). Increased milking frequency may remove FIL and thus reduce its inhibitory effects on milk synthesis. Secondly, decreasing milking frequency results in an increased physical pressure, which may reduce secretory cell volume or cellular hydration states in the mammary gland. Cellular hydration states are regulated by cell swelling and shrinking. Cell swelling activates membrane transport pathways which lead to the net loss of osmolytes and osmotically obligated water, conversely. cell-shrinking activate

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membrane transport system which acts to increase the amount of solutes, and hence water, entering the cell. In mammary cells, cellular hydration is an important cellular signal and is capable of mimicking the effects of some hormones, and protein synthesis is acutely regulated by cellular hydration (Haussinger 1996; Millar et al., 1997). The transduction pathways for both FIL and cellular hydration in the mammary gland are not known. The change of cellular hydration and FIL signals caused by modulation of milking frequency may also be transduced by or interact with Stat5 to regulate milk synthesis.

Milk protein concentration represents the net balance of milk protein gene expression and protein synthesis, and catabolism of milk proteins in the mammary epithelial cells. Transcriptional regulation is thus only one of multiple elements that influence milk protein production. The data presented here indicate no clear relationship between the DNA – binding activity of the transcription factor Stat5 and milk protein production. During late pregnancy in non-lactating animals, Stat5 appears to be active when there is no milk synthesis. When milk protein synthesis was increased by increased frequency of milking, Stat5 activity was elevated, however, when milk synthesis was increased by hormonal injection, Stat5 activity fell. Thus while experiments in transfected cells would appear to suggest a close coupling between Stat5 activity and β-casein transcription, the results obtained here suggest that that is either not the case or that RNA and / or protein degradation are important contributors to net protein secretion rate.

It remains unclear whether Stat5 activity is related to milk protein concentration. The 18 lactating cows from the hormone infusion trial had milk protein concentrations ranging from 2.97 % to 3.24 %, and Stat5 activity was correlated with

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average milk protein concentration ($r=0.5$; $P<0.05$). There was no relationship between Stat5 activity and milk protein yield. In the study of milking frequency, the Stat5 activity as well as milk protein yield were significantly increased by twice daily milking in comparison with once daily milking. However, milk protein concentration was not different between once and twice daily milking during the final three days before biopsy ($3.26 + 0.02$ % vs $3.29 + 0.05\%$, respectively, $n=5$, $P>0.05$).

In conclusion, the Stat5 DNA - binding activity and protein were present in bovine mammary gland throughout lactation. Stat5 activity and protein in mammary tissue appear to be related to hormonal status, milking frequency and time since the last milking. The data support our hypothesis that the signal transduction pathways of different physiological influences converge upon mammary Stat5 *in vivo* in lactating cows. However, the data presented here also indicate that the level of mammary Stat5 activity is not consistently coupled with levels of milk protein production.

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Yang J. Kennelly JJ. and Baracos VE.. Activation of transcription factor Stat5 responds to prolactin, growth hormone and IGF-I in rat and bovine mammary explant culture (submitted), 2000

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CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

Milk synthesis in the mammary gland is a complex physiological process that is not completely understood. One molecular link between lactogenic hormone receptors and the promoters of milk protein genes has recently been established since transcription factor Stat5 was identified in the mammary gland. Analysis of Stat5 activation provides information for the signal transduction pathways of various extracellular stimuli in the mammary cells. The goal of this research was to examine Stat5 activation and its physiological regulation in bovine mammary gland.

4.1. EXPERIMENTAL STRATEGIES

4.1.1 Mammary gland biopsy and explant culture

It has been a challenge to obtain experimental materials to study bovine mammary gland. Establishment of bovine mammary epithelial cell lines has been reported, including MAC-T and BME-UV cell lines. The MAC-T cell line was originally demonstrated to be capable of differentiation and casein synthesis, but subsequent culture has been shown to be phenotype-unstable with erratic casein expression (Huynh et al., 1991; Zavizion et al., 1995). The BME-UV cells are capable of synthesizing low levels of α-lactoalbumin and α_{s1}-casein (Zavizion et al., 1996), however it has not been reported whether the cell line had the capabilities of β-casein synthesis and prolactin responsiveness, which are the basic functions of mammary epithelial cells. Thus, bovine mammary cell lines with a stable function of milk protein synthesis and lactogenic hormone responses are presently still not available.

Mammary explant culture provides an alternative for *in vitro* studies. Mammary tissue samples in adequate amounts are required for tissue culture and well-

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designed analysis. We use mammary gland biopsy to obtain mammary tissue for making explants. The biopsy procedure was originally described by Knight et al. (1992). It requires deep sedation and local anesthesia, and is characterized by rapid recovery without adverse effects on secretory tissue. Animals do not show any signs of resistance provided they are appropriately handled and given a correct dose of anesthesia. Mammary gland normally recovers within a week after surgical intervention. Milk yield returns to a normal level by three days after the biopsy. Tissue samples of 5-10 g can be obtained. Because the surgical procedure is relatively invasive, it is not practical to obtain mammary tissue frequently. In addition, it is necessary to give the animal several weeks to recover. Thus, the mammary explant culture based on biopsied mammary tissue is limited for extensive and repeated tissue culture. It has been revealed that considerable inter-individual variations were present in mammary Stat5 among animal in similar stages of lactation. To investigate the source of some of these variations, multiple longitudinal samples of mammary tissue from the same animals would be necessary. Technological development in mammary gland biopsy is still needed to facilitate this kind of study.

Immediately following the biopsy, obtained mammary tissues are sliced into mammary explants ($1mm^3$) and cultured in medium bubbled with 5%CO_2/95% O_2. In mammary explant culture, differentiated mammary epithelial cells are incubated in chemically defined media, which facilitate control of the cellular environment and eliminate certain problems of analysis that are inherent in experiments performed *in vivo*. The fully developed cells produce specific milk components in the mammary explants for a period of time. They are physiologically normal. In addition, it is a

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three-dimensional culture system and pieces of mammary tissue are incubated in the media, where the mammary epithelial cells are still attached with extracellular matrix and maintained in a structural integrity. Experimental observations obtained from *in vitro* mammary explant culture would be close to what is observed *in vivo* (Dils and Forsyth, 1981).

4.1.2. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) is a method for identification and investigation of DNA-binding proteins. Knowledge of the DNA-binding transcription factor in a tissue type can be an important aid towards a more thorough understanding how the tissue expresses its function (Dent and Latchman, 1993). The EMSA attempts to determine the potential for a gene to be transcribed in a cell type by identifying the presence of DNA binding proteins capable of binding to a promoter. This presents a very simple situation compared with complicated gene transcription process *in vivo*; nevertheless, it is one of the indications of transcription systematic process.

Transcription factor Stat5 specifically binds to the promoter region from -100 to -80 nucleotides of β-casein gene in the mammary gland. The DNA binding activity of Stat5 is determined by EMSA. The binding sequence is critical for detection of a DNA-binding protein. The binding sequence of Stat5 (5′-AGATTTCTAGGAATTCAAATC-3′) is based on bovine β-casein promoter region, and is conserved in several casein genes in rodents and bovines. We have demonstrated that this sequence binds to mammary Stat5 in lactating rats, sows and cows (Fig 4.1). It is known that nucleotide mutations in the DNA sequence at tyrosine position and dephosphorylated Stat5 protein are not able to form Stat5 DNA complex

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in EMSA analysis (Schmitt-Ney et al., 1992; Gouilleux et al., 1994). Therefore, the activity of Stat5 detected by EMSA shows the amount of biologically active Stat5 protein in the tissue. Stat5 activity is quantified based on the density of bands of Stat5-DNA complexes, and Stat5 activities induced by different treatments can be compared with one another within EMSA. This relative quantification of Stat5 activity is important for studying levels of hormonal activation of Stat5, in particular for the mammary explants, which is taken from lactating animals where Stat5 activity is already present in the tissue in some cases before the explant culture. Hormonal induction of Stat5 activation is therefore determined on the basis of comparisons of Stat5-DNA complex band densities between incubated and unincubated mammary tissue. The relative quantification of Stat5 DNA binding activity provides information for comparisons of different treatment if all the samples are loaded in same amount of nuclear protein and run in one pair of plates.

Nuclear protein are separated by SDS-polyacrylamide gel electrophoresis. Proteins are transferred from the gel to the membrane and stained with Ponceau S (Fig. 4.2). The density of the major protein band in the Ponceau S stain is consistent with amount of nuclear protein loading. The bands represented for Stat5-DNA complex in the EMSA were scanned by Imaging Densitometry (Bio-Rad Laboratories). In order to have minimum influences from the background, the density volume for each Stat5-DNA complex was deducted from its own lane background. The data are expressed as the density units of adjusted volume and comparisons between two groups were performed using student t-test within one X-ray film.

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The data from EMSA is useful for analyzing hormonal and growth factor effects on a transcription factor. However, a detection of Stat5 DNA binding activity in the tissue does not completely represent the actual transcription activity of a gene whose promoter contains the binding DNA sequence for Stat5. As noted in the COS cells transfected with GH receptor and Stat5 cDNA, growth hormone induced Stat5 DNA binding activity, but did not result in correspondingly increase in the β-casein-promoter linked reporter gene activity (representing the actual gene transcription activity). Therefore, multiple levels of measurements, including EMSA, translocation of the transcription factor, and transactivation activity, would be required for evaluation of the activity of transcription factors.

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Species:	rat	porcine	bovine

Stat5-DNA
complex

Lane	1	2	3	4	5
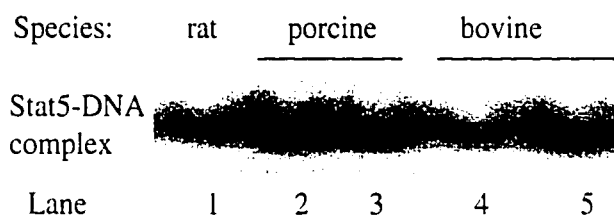
Fig. 4.1. Stat5 DNA binding activity was detected in mammary tissue from lactating rat, sow and cows.

Mammary tissue was taken from slaughtered animals and nuclear extracts were prepared. Stat5 activity was analyzed by EMSA using 4 μg nuclear protein from each sample, where Lane 1, Lane 2 and 3, and Lane 4 and 5 were from rat, sows and cows respectively.

Hormone	Fresh	BCM	BCM	PRL	GH	PRL	GH
Incubation (min)	0	30	60	60	60	90	90

M

Band X

Density of the major band X

17	17	16.6	16.4	17.3	17.9	16
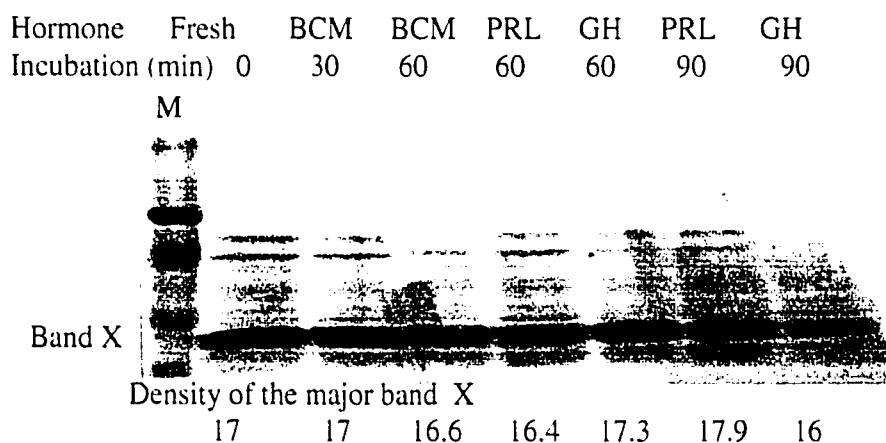
Fig. 4.2. Ponceau S stain of a membrane transferred with nuclear proteins

Mammary tissue was taken from lactating cows and made into explants. The mammary explants were cultured in medium 199 supplemented with 1 μg/ml insulin and 1 μg/ml hydrocortisone and 1 mg/ml bovine serum albumin and treatment hormone of 1 μg/ml prolactin (PRL) or growth hormone (GH) as indicated above each lane. BCM was referred to basic culture medium, which was only supplied with insulin and hydrocortisone. At the indicated times, the mammary explant culture was terminated. Nuclear protein was isloated from the tissue and 10 μg of nuclear protein was separated on the 7.5% SDS-PAGE and the proteins was tranferred to a memebrane, which was stained by Ponceau S soiuiion.

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4.2. STAT5, PROLACTIN, GH AND IGF-I

The data presented in this thesis provide evidence and support the view that Stat5 is involved in signaling pathways of multiple factors related to lactation from both *in vitro* and *in vivo* observations. *In vitro*, Stat5 responds to lactogenic hormones, including prolactin and GH, and IGF-I in the mammary explant culture. *In vivo*, we observed that mammary Stat5 is not only influenced by hormones but also by non-hormonal stimuli such as local mammary changes caused by different milking frequency. In general, different extracellular signals are assumed to be transduced to the nucleus via different pathways to induce specific cellular response. The information accumulated from the Stat5-involvement in multiple signaling pathways provides an example that different pathways interact with each other and could converge at one signaling molecule. Stat5 represents a common route by which different extracellular signals converge and are transduced into nuclei in mammary epithelial cells (Wakao et al. 1994).

Our results in two species provide further evidence that Stat5 plays a role in transduction of hormonal signals in the mammary gland. One unique characteristic of mammary epithelial cells from either rodents or ruminants is to synthesize milk protein such as β-casein, which has not been observed in any other cell types. In the signal transduction cascades of lactogenic hormones, Stat5 is one of the regulatory factors that directly interact with milk protein genes in the nucleus by binding to their promoter, and influence gene transcription. Our results indicate that PRL and GH activate Stat5 in the mammary cells. From the studies of the hormone receptor we know that both prolactin and GH receptors are single-chain proteins with unique

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transmembrane-domains of a proline-rich region termed box1. This region is the site that interacts with Jak2 kinase (DaSilva et al., 1994; Lebrun et al., 1995; Kelly et al., 1993). Both receptors are activated by ligand induced homo-dimerization (Kelly 1993; Goffin et al., 1993). In addition to sharing Jak2 protein kinase by both PRL and GH receptors, the mitogen-activated protein (MAP) kinase pathways have also been reported in different biological systems to be activated under stimulation of PRL and GH receptors (Goffin and Kelly 1996). The MAP kinases are a family of cytoplasmic serine kinases that are activated in response to a diverse array of extracellular ligands, and they act on various cellular substrates including kinases, phospholipases and transcription factors. Prolactin and GH have been shown to induce phosphorylation of extracellular signal-regulated kinase (ERK), a subtype of MAP kinase (Campbell et al., 1992; Wartmann et al., 1996). In the mammary cells, all these pathways about PRL and GH are possible based on the presence of PRL and GH receptors in cells. However, no evidence has been found that GH would also activate milk protein gene expression. Thus the role of activated Stat5 by GH in the mammary cells currently not known, in particular what genes other than milk protein genes would be regulated by GH-Jak2-Stat5 pathways? It is possible that Stat activation via GH could influence other genes in addition to milk protein gene, but deserves further study.

The mechanism by which IGF-I induces Stat5 activation has not been defined in the literature. The IGF-I receptor exists as a α_2-β_2 heterodimer in cells. The ligand-binding domain is located on the extracellular α-subunit, and approximately one-third of the β-subunit is extracellular and is connected to the intracellular portion by a single transmembrane domain. The tyrosine kinase catalytic site is the ATP-binding site that

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is located on the cytoplasmic portion of the β-subunit. The critical events of the binding of IGF-I to the receptor are the activation of the intrinsic tyrosine kinase within the intracellualr β-subunit leading to tyrosine auto-phosphorylation and the tyrosine phosphorylation of cytoplasmic substrates (De Meyts et al., 1994).

Currently it is known that IGF-I receptor signaling pathway leads to the activation of *ras* proteins and series of MAP kinases (Rubin and Baserga, 1995). The *ras* proteins belong to the large *ras* superfamily of monomeric GTPases. They help relay signals from receptor tyrosine kinase to the nucleus by activating MAP kinase pathway cascades (Lowy and Willumsen, 1993). Like most growth factors, IGF-I receptor does not activate *ras* directly, rather recruits *ras* activating molecules such as Insulin-related substrate-I (IRS-I) and Src-homology/collagen (Shc). Both IRS-I and Shc have several SH domains, which interacts with IGF-I receptor, could activate Ras proteins, thereby resulting in activation of series of MAP kinases (Myers et al., 1993; Giorgetti et al., 1994). It has been shown in CHO cells that inhibition of mitogen-activated protein kinase (MAPK) kinase could abolish the GH-induced MAPK activity as well as Stat5 activity (Pircher, et al., 1997). Therefore, in CHO cells the MAPK pathway interacts with Jak/Stat pathway at the level of Stat5. We speculate that IGF-I might induce Stat5 activity through a protein kinase activated by IGF-I receptor or its phosphorylated substrates such as IRS or Shc. Experiments using co-transfection of IGF-I receptors and Stat5 cDNAs and specific inhibitors of IGF-I associated phosphorylation will help to clarify this pathway of IGF action.

4.3. STAT5 AND β-CASEIN GENE TRANSCRIPTION

Transcription of a gene is controlled by its promoter and multiple trans-acting factors binding to the promoter (Alberts et al., 1994). Studies in transgenic mice and transfected cells have revealed that proximal genetic regulatory elements in the β-casein promoter are required for the appropriate regulation of β-casein gene expression (Rosen et al. 1989). Deletion and site-specific mutational analysis of the β-casein proximal promoter have defined a minimal ~100-bp region necessary for proper hormonal regulation of a heterologous reporter gene (Wakao et al., 1992). Within this region, milk box consensus occurs at -140 to -110 and has been shown to repress reporter gene expression (Laird et al., 1988). Deletion or site-specific mutagenesis of the milk box region led to increased basal activity of a β-casein promoter-CAT construct (Schmitt-Ney et al., 1991). The trans-acting factor interactive with this region was characterized to be the ubiquitous nuclear factor YY1 (yin and yang 1, Meier and Groner, 1994; Raught et al., 1994). In the mouse epithelial cell line HC11, YY1 binds to the milk box of β-casein gene promoter and inhibits gene transcription. The YY1 consensus binding site is conserved in the rat α_{s1} -, β- and γ-casein (Yu-Lee et al., 1986)

Stat5 binds to the DNA sequence between -80 to -100-bp in the β-casein gene promoter. The DNA binding sites of Stat5 and YY1 are adjacent each other and possibly overlapping. Studies in HC11 cells have suggested that Stat5 may act through the displacement of YY1, therefore, relief of YY1 repression, and results in positive influence on β-casein gene transcription (Groner et al. 1994). It was found that β-casein synthesis under the control of lactogenic hormones was unrelated to YY1

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concentrations even though the activity of YY1 was increased by 5-10 fold through introduction of a retrovirus vector of YY1 in HC-11 cell culture. During hormonal induction in HC11 cells, Stat5 was activated but the concentration of YY1 did not change (Meier and Groner 1994). The EMSA analysis showed that the incubation of the YY1-DNA complex with Stat5 did not result in a ternary complex comprising Stat5, YY1 and DNA, but led to the displacement of YY1 and the formation of Stat5-DNA complex (Meier and Groner 1994). These findings demonstrate that Stat5 may positively regulate β-casein gene transcription by relief of YY1 repression.

On the basis of the molecular mechanism of Stat5 action on β-casein gene transcription, increased mammary Stat5 activation by extracellular signals would stimulate β-casein gene transcription, possibly resulting in increased β-casein mRNA accumulation. Transcriptional activity under condition where Stat5 activity varied was not measured here and is an important limitation of this work. It is not known how much the stimulated β-casein gene transcription by Stat5 will contribute to the total β-casein mRNA pool in mammary epithelial cells. In a preliminary study, we measured β-casein mRNA levels in rat mammary explant tissue (Fig. 4.3). Under the hormonal treatments of PRL and GH for 1-h incubation, the β-casein mRNA levels were very similar even though the Stat5 activity was markedly changed by the hormonal induction (Chapter 2). This either proves or disproves a realtionship between Stat5 and β-casein transcription because mRNA pool size may be too large and not rapidly modulted on this incubation time scale. The levels of casein mRNA are also influenced by their catabolic rate. In our *in vivo* studies, there appeared to be no strong association between Stat5 activity and milkp protein synthesis. Mammary Stat5 in

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late-pregnant and non-lactating cows appeared to be active when there was no apparent ongoing milk synthesis. When milk protein synthesis was increased by increased frequency of milking, Stat5 activity rose, however, when milk synthesis was increased by hormonal injection, Stat5 activity fell. In the pooled data from 18 lactating cows, the relative Stat5 activity was correlated at a middle level with average milk protein concentration (r=0.5), but was not correlated with milk protein yield. In the study of milking frequency, the Stat5 activity, as well as milk protein yield were significantly increased by twice daily milking in comparison with once daily milking. However, milk protein concentration was not different between once and twice daily milking during the final three days before biopsy (Chapter 3). The increased Stat5 activity by twice daily milking did not result in an additional increase in milk protein concentration. Others have also reported that Stat5a-deficient transgenic mice showed no full lobuloaveolar development, but β-casein gene was expressed in the mammary tissue, suggesting that Stat5 is not mandatory for β-casein gene expression (Liu, et al., 1997). Therefore the essential role of Stat5 DNA binding activity for casein gene expression, demonstrated by *in vitro* COS cell transfection is questioned. Factors other than Stat5 may play critical roles in controlling casein gene expression in the mammary gland during lactation.

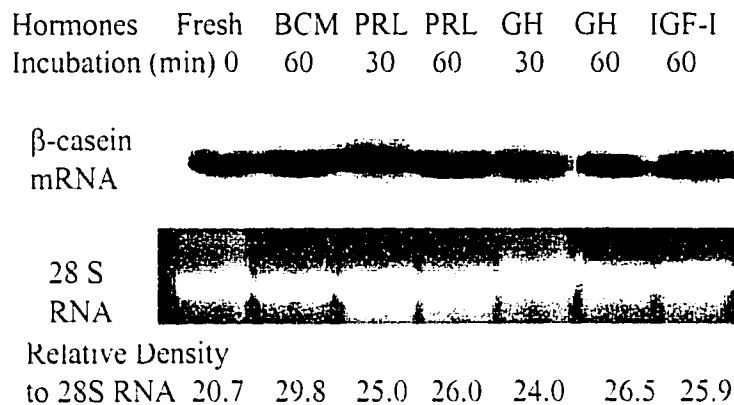In addition, the biological control of milk protein synthesis does not appear to occur only in initiation of gene transcription in the mammary gland. For example, earlier observations in lactogenic induction of β-casein gene transcription found that transcription rate was only increased two-fold by lactogenic hormones, while the mRNA stability, measured by half-life of the mRNA increased by 20-fold by

lactogenic hormone treatment over the control (Guyette et al., 1979). Therefore, post-transcriptional regulation of milk protein gene makes important contributions to the level of steady-state milk protein mRNAs. It is not known how the lactogenic hormones regulate the mRNA stability of milk proteins. In primary cultures of rat mammary epithelial cells, α-casein mRNA was shown to be primarily regulated on post-transcriptional levels by laminin, a major constituent of the basement membrane. Measurements on the turnover of α-casein mRNA show that this mRNA is stabilized four-fold more on laminin than on tissue culture plastic (Zeigler et al., 1992). In mammalian cells, mRNAs display substantial differences in their susceptibility to degradation by ribonucleases. It is known that mRNAs encoding some cytokines are very unstable in resting cells, but once cells are activated, the same mRNA are relatively stable, decaying in some cases with half-lives greater than 4 h (Rajagopalan and Malter 1997). Studies in cytokine mRNA decays have identified some mRNA binding proteins that interact with the AUUUA motifs of the mRNAs to prevent mRNA degradation (Gillis and Malter 1991). The biological control of milk protein gene expression may not act exactly in the same way as cytokines mRNA. However, multiple steps of controlling mechanisms, including initiation of transcription, RNA processing and transport, RNA degradation, protein synthesis and degradation, certainly give greater regulatory flexibility in response to changing lactogenic hormones and nutrient supplies to the mammary gland.

In the data presented in this thesis, studies about Stat5 have primarily focused on hormonal and non-hormonal induction of Stat5 activity without consideration of the resulting β-casein gene transcription and protein synthesis. Thus, the results are

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useful in the understanding of the upper stream of hormone signal transduction, but limited in lack of actual measurement of downstream effects in gene transcription. The actual gene transcription is not only controlled by downstream events such as transcription factor transactivation, but also regulated by multiple transcription factors and their interactions. Further studies are needed to investigate whether induced Stat5 by extracellular signals from hormonal and non-hormonal stimuli will result in an increased β-casein transcription and final mRNA accumulation and milk protein in the mammary gland.

In dairy production, milk protein synthesis is also significantly influenced by nutrients such as amino acids and energy sources availability in the mammary gland. Milk protein synthesis starts with lactogenic hormone stimulation to the mammary gland. If the transduction pathways of the hormones through transcription factors of Stat5 and YY1 and β-casein mRNA accumulation more likely are one of the mainstreams for mammary cell function during lactation, how do these signal network respond to the adequate or inadequate nutrient supplies? Therefore the system of explant culture and Stat5 measurement established by this thesis only partially provides some information on hormonal response in the mammary gland, majority work in nutritional manipulations of milk protein and interactions with hormone signaling pathways are still needed for future studies.

Hormones	Fresh	BCM	PRL	PRL	GH	GH	IGF-I
Incubation (min)	0	60	30	60	30	60	60

β-casein
mRNA

28 S
RNA

Relative Density							
to 28S RNA	20.7	29.8	25.0	26.0	24.0	26.5	25.9

Fig. 4.3. Effects of hormonal treatments on β-casein mRNA in rat mammary explants.

Mammary tissue was immediately taken from mid-lactating rats and made into explants. The mammary explants were cultured in medium 199 supplemented with 1 μg/ml insulin and 1 μg/ml hydrocortisone and 1 mg/ml bovine serum albumin and 200 ng/ml prolactin (PRL), 50 ng/ml growth hormone (GH) or 50 ng/ml IGF-I. BCM was referred to basic culture medium, which was only supplied with insulin and hydrocortisone. At the indicated times, the mammary explant culture was terminated. 20 μg of total RNA isolated from the explants were used for detecting mammary β-casein mRNA by Northern blotting. The X-ray autoradiogram was exposed for 48 hours.

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4.4. CONCLUSIONS

These studies begin to elucidate molecular physiology of the mammary gland. Transcription factor Stat5 responded to prolactin, growth hormone, and IGF-I in rat and bovine mammary explant cultures. Growth hormone at 2-5 times of physiologic concentration independently induced Stat5 activation in bovine mammary explants. Investigations of physiological regulation of mammary *in vivo* Stat5 reveal that Stat5 is present in late pregnancy and lactation, and regulated by hormonal treatment and mammary local changes induced by varying milking frequencies. The results generally indicate that Stat5 mediates multiple hormonal and growth factor signals and serves as a common point in the signal pathways of the various lactogenic hormones and non-hormonal stimuli in bovine mammary gland. Insight is provided into Stat5 involvement and interactions with other signal regulatory molecules in the mammary gland. The dominant role of Stat5 activation in milk protein gene expression is questioned and it is identified that this is the area that deserves further detailed study.

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APPENDIX A. MAMMARY EXPLANT CULTURE

Explant culture is an *in vitro* incubation of fragments of tissue transplanted from its original site into an artificial medium. Mammary explant culture is different from primary cell culture, where dispersed cells are obtained by protease digestion of connective tissue and grown as sheets on a suitable support. Explant culture is a three-dimensional culture system. It does not separate the cells from their support matrix and keep cells alive in a manner of tissue-structural integrity. In the case of mammary tissue, the mammary epithelial cells are connected to basement membrane and around the lumen. It is very important to keep tissue integrity during mammary culture because the normal function of mammary epithelial cells largely depends upon their extracellular matrix. Yet, there are some limitations in explant culture in terms of experimental convenience and culture technical requirements. Explant culture can not be propagated and maintained for a very long time, thus each experiment requires recourse to the original donor tissue. It is difficult to make uniform explants by current technology, which is crucial to decrease variations on the measurements of biochemical and molecular criteria on the tissue. The following describes general procedure requirements for mammary explant culture in rats and cows.

Culture Media

Chemically defined synthetic media have been successfully used for mammary explant culture. Medium 199, supplemented with Hanks' or Earles's salts mixture, are one of the most commonly used culture media. Penicillin G and streptomycin are added to a final concentration of 35 μg/ml and 10μg/ml respectively. Sterilization of

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the media is effected by passage through Millipore filter (0.45μM) contained in vacuum pump- operated filter unit.

Hormones

Addition of insulin, a glucocorticoid and prolactin to the culture system promotes the conversion of non-secretory epithelial cells, in explants of mammary tissue derived from animals in various developmental stages, into secretory cells. They also maintain the function of mammary cells in the explant tissue obtained from lactating animals. These hormones have been shown to produce their characteristic effects when used at levels between 10^{-9} and 10^{-8} M. They are usually employed at considerably high concentration, i.e. 1 μg/ml each. This might be due to concerns about hormone absorption onto glassware and the low biological activity of extracted hormones and imperfect *in vitro* culture conditions for mammary tissue.

Insulin stock solution (2 μg/μl): Dissolve insulin in 5 mM HCl solution.

Prolactin stock solution (2 μg/μl): Dissolve prolactin in 0.1 mM NaOH solution.

Hydrocortisone stock solution (1μg/μl): Dissolve hydrocortisone in anhydrous ethanol.

Tips: When hydrocortisone is added to the culture media. The final concentration of ethanol in the media should be no more than 0.5%. The working concentrations of the hormones are suggested to be 1-5 μg/μl for insulin. 0.5-5 μg/μl prolactin and 1 μg/μl hydrocortisone.

Preparation of Mammary Tissue

Stainless steel microsurgical instruments for making explants includes

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- Curved forceps, 11-cm, suitable for handling explants in-groups.

- Watchmaker forceps, 11.5-cm, the sharp points and the curved forceps make it suitable for dissection and for handling individual explant.

- Spring scissors, 8.5-cm with 1-cm blades, suitable for cutting explants.

Normally, mammary tissue, ductal-alveolar and lobular-alveolar mammary tissue, is obtained from adult non-pregnant, pregnant and lactating mice or rats. These developmental steps may be prepared by appropriate hormonal treatment of virgin females. There are five pairs of mammary glands in rats or mice. The second and third glands have usually been selected for culture because of their relative flatness and ease of dissection. Note that the second thoracic gland is partially obscured by a thin strip of muscle, which should be dissected before attempting to remove the mammary gland.

Female rats are usually killed by cervical dislocation, washed in 70% ethanol, and pinned the feet to a corkboard or similar working surface. The glands are exposed by making a mid-ventral incision along the length of the body, and loosening and pinning the skin to the corkboard. After removing the mammary tissue, put the tissue in the medium 199 supplemented with 1 mg/ml bovine serum albumin. Portions of fat pads are removed through cutting along their edges with iridectomy scissors or a sharp scalpel. Large portions of mammary can be sliced into thin sections with razor blades or a tissue slicer. The sections may then cut into thin strips until finally cubes or blocks of the desired dimensions with scissors or a microtome. Mammary explants favorable for culture are approximately 1.0 mm thick and 1-2 mm in diameter. It is

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very important to keep the tissue always wet in proper media. Drying the tissue is detrimental to the cellular normal metabolism in the following tissue culture.

Bovine mammary tissue can be obtained from lactating or late-pregnant cows by surgical biopsy. The procedure of bovine mammary gland is carried out as described by Knight et al (1992) with the following modifications. Briefly, an intravenous catheter (14g-5.25 inches) is placed in the jugular vein. Deep sedation of the cow is achieved by slow intravenous injection of xylazine hydrochloride (5 mg/50 kg body weight, approximately 50-70 mg per cow), alternatively ketamine (2 mg/kg body weight) is supplemented. Animal is then tipped and held recumbent on their right side. The feet are restricted for safety and to allow clear access to the udder. The animal is maintained on a glycerol guaiacolate (GGE)/ketamine drip, containing 50 g GGE, 1000 mg ketamin, 1000 ml 50% Dextrose, 900 ml sterile water, for the duration of the surgery. The drip rate is set at 20 mLs per minutes. Additional xylazine is given during surgery (50-100 mg) if further relaxation is required. The left side of the udder is shaved and then cleaned with aseptic surgical scrub. The biopsy site is selected in the basal (upper) portion of the udder, avoiding fat and large subcutaneous blood vessels wherever possible. Then skin is swabbed with 70% ethanol and iodine surgical scrub. Local anesthesia is achieved by line block subcutaneous injection of 5-10 ml 2% lignocaine hydrochloride. A skin incision 8-cm long is made through the skin. The capsule is identified with blunt dissection. The mammary tissue is exposed with blunt dissection and a 5-10 g of portion of mammary tissue is removed by scalpel. Homeostasis achieves by suturing and ligation using 3 metric catgut. The surgical cut is closed with 4 metric catgut suture in the deep tissue and skin closure is achieved

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with suture using #1 Vetafil in a Ford interlocking suture pattern. Cows are treated with Ethacillin-Procaine penicillin G (300,000 IU/ml, Pfizer) 2 ml/45 kg body weight twice daily for 5 days, and Cefa-lak (200 mg cepharin sodium, Ayerst Laboratories) intra-mammary for three consecutive milkings following the surgery.

Culture vessels and Conditions

To maintain explants at the surface of liquid media, it is convenient to employ raft support of stainless mesh, nylon or lens papers. Lens paper can be placed on top of stainless steel grids, or as is required for most of the polyester fabrics, treated with silicon to allow them to float without support. In these cases, vessels with loose-flip tops such as petri-dish are suggested. Alternatively, the explant tissue can be cultured in temperature and gas supply-controlled incubator. The gas mixture of 5% CO_2/95% O_2 is normally used.

We have employed mammary explants in 14 ml Falcon tube bubbled with 5% CO_2/95% O_2 in a water-circulated incubator. With the gas bubble in the tube, the mammary explants are able to float, making mammary tissue easy to access to medium constituents and gases.

OPERATIONS

- Wash the mammary tissue twice with medium 199 in a petri-dish.

- Cut away the connective tissue with fine scissors.

- Cut tissue into small pieces with scissors and scalpel, making explants ($1-5$ mm^3).

- Add approximately 1 gram of tissue into the tube with 10-ml culture media.

- Add hormones or agents into tubes according to the treatment designs.

- Supply gas mixture (95%O_2/5%CO_2) to the tube and incubate the explant at 37°C.

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- Upon completion of tissue culture, centrifuge briefly and collected the tissue and medium (if desired) and quickly freeze the samples in liquid nitrogen and stored in- 80°C for future analysis.

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APPENDIX B. ELECTROPHORETIC MOBILITY SHIFT ASSAY

With recombinant DNA technology, researchers have isolated numerous biologically important genes. It is known that DNA-binding proteins such as transcription factors play a major role in gene expression through their involvement in transcription, replication and recombination. Electrophoresis mobility shift assay (EMSA) was developed to detect the activity of DNA-binding proteins and widely used for understanding how transcription of these genes is controlled in response to environmental or developmental signals. The techniques of EMSA are based on the observation that a DNA-protein complex migrates more slowly than "free" DNA during non-denaturing gel electrophoresis. In a typical EMSA, a ^{32}P-labelled DNA fragment containing the putative binding target site is incubated briefly with cell extracts and is then subject to electrophoresis in a non-denaturing gel. If proteins present in the extract bind to the target sequence on the labeled DNA fragment, the fragment is "retarded" or "shifted" in its migration through the gels, and appears in subsequent analysis by auto-radiography to be of higher molecular weight than free DNA (Fig.A.1). Therefore EMSA is also simply referred to "gel-retardation" or "band-shift" assay. The following description of EMSA protocol will be divided into three procedures: preparation of nuclear extracts, radio-labeling target DNA and detection of DNA-protein interaction.

Procedure A. Preparation of Nuclear Extracts

Protein extracts may be prepared from whole cells or isolated nuclei. The nuclear extract is mostly used in EMSA and the following will use mammary tissue as an example to describe the operation procedures of preparation of cell nuclear extracts.

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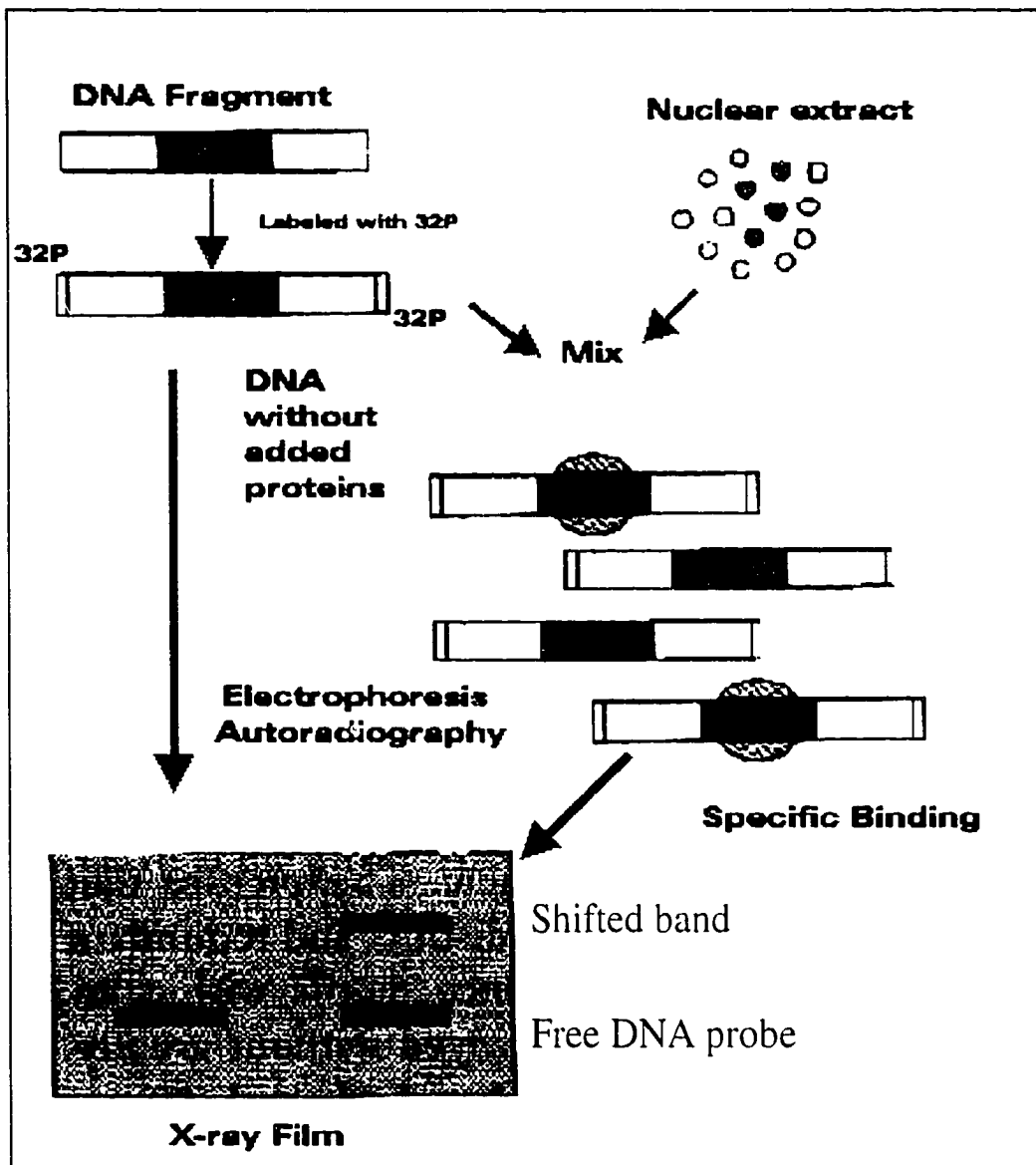
Fig. A1. Electrophoresis Mobility Shift Assay

The DNA sequence specifically for a nuclear protein is labeled with ^{32}P-dNTP.
Binding of the nuclear protein to the labeled DNA causes it to move more slowly than
the DNA alone upon gel electrophoresis, and hence results in the appearance of
retarded band upon autoradiography to detect the radioactive level.

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Lysis Buffer (10 mM Hepes, 25 mM KCl, 0.5 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2M Surcose, 10 % glycerol, pH 7.6).

To make 1000 ml

>2.383 g Hepes
>1.864 g KCl
>2ml 0.5 M EDTA
>5 ml 30 mM spermine
>5 ml 50 mM spermidine
>684.6 g Sucrose
>100 ml glycerol

Tips: It takes about 2-4 hours to dissolve the sucrose because of its high concentration.

A heavy strong magnetic stirring instrument may facilitate the dissolving process.

Extraction buffer (400 mM NaCl, 1 mM EDTA, 20 % glycerol, pH 7.9)

To make 100 ml

>0.477 g Hepes
>2.34 g NaCl
>200 µl 0.5M EDTA
>20 ml Glycerol

The following chemical agents will be added to the extraction buffer according to the indicated working concentrations before using the buffer.

>0.1% Triton X-100
>1 mM DTT
>0.2 mM PMSF (phenylmethylsulfonylfluoride)
>5 µg/ml leupeptin.
>2 µg/ml pepstatin
>5 µg/ml aprotinin

OPERATIONS

- Grind 0.4–2 g of tissue in a pre-cooled (–80°C) mortar and pestle in liquid nitrogen.

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- Transfer the tissue powder into a 14 ml round bottomed Falcon tube (Becton Dicknson, Franklin lakes, NJ, USA) and stored in –80 °C freezer.

Tips: Try to store the mammary tissue powder in a short period of time (<1 week) in order to prevent proteins degradation or loss of their biological activity during storage.

- Homogenize the tissue in 5ml lysis buffer using a polytron power homogenizer at low speed for 30 s in the Falcon tube in order to dissociate the tissue.

Tips: Samples should be kept on ice at all times and centrifugation should be carried out at 4 °C.

- Homogenize the dispersed tissue on ice with 20 strokes in a 7ml tight-fitting Dounce tissue grinder to release the nuclei (Weaton Scientific USA).

- Centrifuge the homogenate at 100,000 X g for 20 minutes on a cushion of the same lysis buffer.

Tips: The nuclei are pelleted through the cushion and precipitate to the bottom of the tube. Successful release of nuclei may be checked by phase-contrast microscopy. Carefully remove the supernatant with vacuum pipette without taking out the nuclear precipitates.

- Re-suspend the nucleus pellets in extract buffer(100-500 μl) and use a pipette to facilitate the suspension.

- Transfer the suspended mixture into a micro-centrifugation tube and gently shake for 20 minutes on ice.

- Centrifuge the suspended nuclei at 10,000 X g for 5 minutes (or 12,000 rpm in micro-centrifuge) in 4 °C.

- Transfer the supernatant containing the nuclear protein into a new micro-centrifugation tube, quickly freeze in liquid nitrogen and then store at –80 °C until EMSA.

Tips: In order to avoid repeatedly thawing the nuclear protein, it is necessary to aliquot in convenient amounts for protein assay and EMSA. If nuclear proteins with relatively high purity are required from a large amount of tissue, it is suggested to have a further dialysis twice for 90 minutes against 250 ml dialysis buffer (25 mM Hepes pH 7.6, 40 mM KCl, 1 mM DTT, 10 % glycerol).

Procedure B. Radiolabeling Target DNA

The DNA probe for EMSA is based on double-strand DNA sequence. The sequence of DNA varys with interested DNA-binding proteins. It could be a restriction fragment or a synthetic oligonucleotide. The size of the fragment is normally kept below about 250 base pairs to have a clear distinction of the probe from other interactive proteins. It is a common practice to have a short DNA sequence (~20 nucleotides) synthesized by oligonucleotide synthesizer. Then the double-strand DNA is obtained by fill-in reaction with Klenow fragment. The method of probe-labeling described below is also suitable for DNA fragments with 5' overhanging ends produced by restriction enzyme. The DNA probe for Stat5 binding is designed based on bovine beta-casein promoter region (-80--100), 5'-AGATTTCTAGGAATTCAATC-3'. One strand of full length of this sequence and its partial complementary oligonucleotide (5'-GATTTGATT-3') are synthesized by oligonucleotide synthesizer. Then the two strands of DNA was hybridized at 55 ° C for 10 minutes. The double-stranded labelled DNA probe is obtained by fill-in reaction

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with [α-^{32}P]dATP using DNA polymerase I Klenow fragment. Normally 0.1-5 ng of DNA (labeled to ~10,000 cpm) per reaction is needed for efficient DNA labeling.

Combine in order in a sterile micro-centrifuge tube

Ultrapure water	29 μl
Target DNA (10 μg /μl)	5 μl
Labeling mix-dATP	10 μl
Klenow fragment	1 μl
[α-^{32}P] dATP	5 μl
Total volume	50 μl

Tips: The agents used in the reaction are from the bandshift kit of Pharmacia Biotechnology (Cat # : 27-9100-01). Labeling mix dATP contains 0.1mM each of dGTP, dCTP and dTTP in 50 mM Tris-HCl (pH 7.5), 50 mM MgCl$_2$, 250 mM NaCl and 25mM β-mercaptoethanol. Klenow fragment is in buffered glycerol solution (5-10 unit/μl).

OPERATIONS

- Mix gently, centrifuge briefly, then incubate the reaction at 37 °C for 1 h.

- Dilute the sample by adding 50 μl of ultra-pure water.

- Purify the ^{32}P-labeled DNA using Sephadex G50 spin column*.

- Pipette the probe out of the eppendorf tube into a new one.

- Count the probe radioactivity via scintillation counter by taking 1μl probe in 5 ml distilled water in a middle-size vial. The reading is between 20,000-100,000 cpm:

- Store the DNA probe at –20°C for about 1-2 weeks

* Making Sephadex G-50 Spin Column

- Dissolve Sephadex G-50 (medium, Pharmacia Biotech) in distilled sterile water (10 g of dry powder yields 160 ml of slurry).

- Wash the swollen resin with distilled sterile water several times to remove soluble dextan.

- Equilibrate the resin in TE (pH 7.6) by 2-3 times.

- Autoclave the suspension and store at 4 °C.

- Fill the bottom of 1 ml syringe with baked glass wool (siliconized) and Sephadex G50 resin up to 0.9 ml.

- Spin the syringe in 14 ml tube at #5 (2000-3000 rpm) for 4 minutes in desktop centrifuge.

- Add Sephadex G50 to top up and spin again for 4 minutes.

- Wash twice (100 ul 1X TE, pH 7.6) by spin at speed #5 for 4 minutes each.

- Then the spin column is ready for purify the labeled DNA probe. The DNA probe is added to the column and spin at #5 for 4 minutes.

Procedure C. Detection of DNA-Protein Interation

We use the 20 minutes incubation at room temperature, whereas others favor incubation on ice for longer time.

10 X binding buffer (100 mM Hepes, pH 7.8, 500 mM KCl)

To make 100 ml
    2.383 Hepes
    0.932 KCl

Tips: dissolve the chemicals with Millipore sterile water and adjust pH to 7.8, sterilize by passing through Millipore filter (0.45 µM), aliquot and store at -20 C.

The binding solution for Stat5 in 20 µl contains 10 mM Hepes, pH7.8, 50 mM KCl, 5 mM $MgCl_2$, 10 % glycerol, 5 mM DTT, and poly (dI-dC) (1µg poly(dI-dC)/ µg nuclear protein). Other chemicals used for the binding reaction are from the bandshift kit (Pharmacia Biotech)

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Add in a sequential order

50% Glycerol	4 µl
10 X binding buffer	2 µl
DTT (50 mM)	2 µl
$MgCl_2$ (100 mM)	1 µl
Nuclear extract (1 µg/µl)	2 µl
Poly (dI-dC)	2 µl
Ultrapure water	5 µl
^{32}P target DNA	2 µl
Total Volume	20 µl

OPERATIONS
- Mix the solution and briefly centrifuge.

- Incubate the solution for 20 minutes at room temperature.

- Add 2 µl of loading dye to each reaction, mix gently and centrifuge briefly.

- Loading the samples to 4% polyacrylamide gel

- Perform eletrophoresis at 10 V/cm with 0.25 X TBE.

- Stop eletrophoresis when the bromophenol blue marker dye has run about two third of the way down the gel.

Tips: The gel should be pre-run at 50V for about 2 hr before eletrphoresis (The current will drop from 20-30 mA to approximately 10 mA during this time).

- Disassemble the gel cassette from the electrophoresis chamber, and carefully slide the spacers out from between the plates without tearing the gel. Using a flat patula, gently pry apart the gel plates, making sure the gel adhere to one of the plates.

- Place the plate with gel in prepared fixing solution (5% methane, 5 % glacial acid) for 5 minutes to fix the gel.

- Lift the gel onto filter paper by firmly applying the filter paper over the top of gel and remove from glass and put another filter paper to back it.

- Cover the gel with Sarah wrap and dry down for 30 minutes in a gel dryer connected with vacuum pump.

- Expose the dried paper and gel to X-ray film for several hours or days at −70 °C using an intensifying screen.

Procedure D. Quantification of Stat5 DNA Binding Activity

The relative quantification of Stat5 DNA binding activity provides information for comparisons of different treatment if all the samples are loaded in same amount of nuclear protein and run in one pair of plates. In order to validate the measurement of nuclear protein and sampling loading, it is necessary to use a Western blotting minigel to run the sample first. After proteins are transferred from the gel to the membrane, a method of protein staining such as Ponseu S stain could be used to evaluate the sample loading. Normally if the samples from one experiment are carried out together in one time of nuclear isolation and protein measurement, the protein amounts are constant in Pouseu S staining with careful sampling (Fig. 4.2).

In the EMSA, a same amount of nuclear protein was added in the binding reaction for all the sample in one pair of plates, and binding reactions were carried out in same condition, which can be easily achieved by making a master solution. When the gel was exposed to an X-ray film, it is necessary to have several repeats with different times of exposure in order to have an overall and clear picture to show all the

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DNA-protein complex for each treatment. Practically, in the case of Stat5 activity, an over-exposed film would not be useful for comparing the different activity of the Stat5-DNA complex from different treatment.

Bands represented for Stat5-DNA complex in the EMSA were scanned by Imaging Densitometry (Bio-Rad Laboratories). The density unit of volume (OD*mm*mm) can be obtained for each band from the analysis. In order to have minimum influences from the background, the density volume for each Stat5-DNA complex should be deducted from its own lane background. The data are expressed as the density unit of adjust volume form treatment and comparisons between two groups were performed using student t-test within one X-ray film.

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