Identification of constraints to bovine milk protein synthesis

P.A. Sheehy, K.R. Nicholas[1], J.M. Gooden, and P.C. Wynn

Department of Animal Science, University of Sydney, Camden, NSW 2570
'Victorian Institute of Animal Science, Attwood Victoria 3049

Summary

Our inability to alter significantly the concentration of protein in milk by manipulating the nutritional status of the cow has provided the impetus for the investigation of rate limiting factors for protein synthesis. One of the major rate limiting factors for these studies has been the lack of an immortal cell line expressing bovine caseins. We have therefore established a method for the biopsy of mammary epithelial tissue from the late–pregnant cow with which to study regulatory mechanisms for casein and whey protein gene expression. Initial studies have identified a period of 30–40 days from calving in which biopsied mammary tissue can be induced to express mRNA for both casein and whey proteins. This model has been used to demonstrate that the stress-induced peptide hormone, bendorphin is part of the inhibitory complex of factors that suppress milk protein expression. These studies suggest that the nutritional management of the cow during this period ***pre-calving*** may be important in minimising the impact of these factors on productivity during the subsequent lactation.

Introduction

Milk is a complex biological resource comprised of many components serving numerous essential physiological functions in both the calf and the cow. In view of their importance, it is not surprising that when we attempt to alter the function of the bovine mammary gland to boost milk production and manipulate the relative proportions of each of the major constituents, we are unable to achieve the desired goals.

The major constituents of milk and their functional significance

Milk is comprised of protein carbohydrate, fat, vitamins and minerals expressed in concentrations that are delicately balanced to optimise the growth of the calf. The casein micelle itself requires the co-ordinated expression of $\alpha s1$, $\alpha s2$, β and κ casein which are held together through hydrogen and disulphide bonding to form a suspension with calcium (Figure 1). Failure of this function would undoubtedly lead to the precipitation of calcium phosphate within the mammary gland thereby making the process of milk letdown a painful experience for the cow.

It is important to recognise, however, that it is not only the nutritional status of the calf that is satisfied by the provision of milk for the calf but also the need for hormones, growth factors and biologically active peptides derived from milk proteins. Thus, although the neonate is dependent on its own physiological mechanisms for survival and growth, a maternal influence is maintained post-partum through the

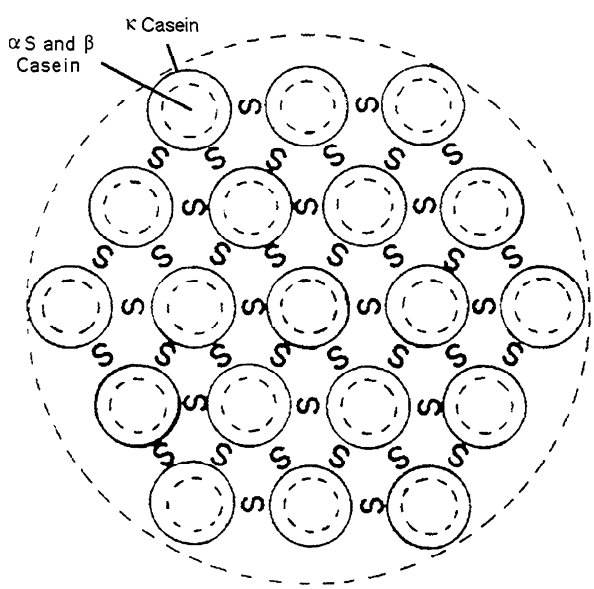

Figure 1 The basic structure of the casein micelle

provision of this complex endocrine cocktail, which plays an important role in various developmental and maturational processes in many organs including the gastro–intestinal tract and the brain of the calf.

Nutritional manipulation of milk synthesis

Australian dairy farmers have often been criticised for under-feeding their milking herds particularly as the industry is based on feeding pasture which is at times poor in quality. The random approach to the provision of concentrate supplements to cows maintained at pasture has often resulted in imbalances in protein and energy in the rumen. This compromises the efficiency of the supply of nutrients to the circulation for use by the mammary gland as the ruminal microflora attempt to maximise the efficiency of synthesis of microbial protein (for further detail see Lean and Westwood in this volume).

The importance of maintaining high nutrient intakes early in lactation in order to boost the total productivity of the lactation has been well established (see Haresign, 1979). Our increased understanding of ruminal fermentation and the importance of the component of the diet that is resistant to degradation by the rumen to the nutritional status of the cow has resulted in marked responses in milk yield. Similarly, the maintenance of good body condition at calving is important as the animal passes through a phase of negative energy balance early in lactation as feed intake fails to meet the metabolic demands of lactation.

Studies involving the dietary supplementation of cows prior to calving have generally aimed at establishing body condition on the pregnant cow in the face of an increasing nutrient demand from the foetus during the latter stages of pregnancy. Broster (1971) reviewed a number of studies conducted by his group and others showing that the time honoured practice of 'steaming up' cows by supplementing diets with concentrates in the weeks before calving resulted in positive but variable responses in the subsequent lactation. These studies were notable for the variation in the nature and quality of the basal diet and concentrates and for the duration of the period of supplementation *pre–partum* which often extended up to 8 weeks prior to calving.

The potential for the manipulation of the cow prior to calving to improve the lactational performance was recognised in the publications of Boutflour over a period of 40 years, who showed that milking prior to calving gave a significant production response (Boutflour, 1967). It is interesting that these early observations have been supported by the recent study of Fowler et *al.* (1991), who demonstrated that continuous milking of a gland in the previous lactation resulted in greater milk yields in the subsequent lactation than that from a gland which had been dried off in the previous lactation prior to parturition.

The metabolic manipulation of milk protein expression

Dietary manipulation has the potential to increase milk protein production faster than the genetic selection of dairy cows but, unfortunately, increases in crude protein content of the diet or even the direct supply of amino acids post–ruminally have resulted in small and variable effects on the concentration of protein in milk (see De Peters and Cant, 1992; Metcalf et *al.* 1992; Sutton, 1992). All of these studies, however, have involved the supplementation of lactating animals with little regard to the fact that the initiation of milk protein expression occurs well before parturition.

However in tissue collected more than 20 days prior to calving and maintained in the presence of I and F, prolactin was able to reverse the decrease in casein and b lactoglobulin gene expression irrespective of whether it was added after 2 or 4 days in culture. Furthermore the addition of these hormones to tissue just collected (time 0) further stimulated the basal level of expression (Figure 4). The timing and magnitude of these responses were not affected by the duration from the involution of the gland from the previous lactation.

Similarly most studies involving the administration of anabolic endocrine agents such as growth hormone, prolactin and insulin like growth factor 1 (IGF1) to stimulate lactation have focused on the treatment of lactating animals (Plaut et *al.* 1987, Bauman, 1992, Bauman and Vernon, 1993 and Prosser *et al.* 1990). None of these treatments resulted in any marked change in the expression of milk proteins.

Milk protein expression and mammogenesis

The normal lactation cycle is associated with a period of exponential growth of mammary parenchyma during the 6 weeks preceding calving, which continues to peak lactation followed by a gradual regression in the size of the gland as lactation proceeds until involution. At this time there is a marked loss of tissue involving the deletion of secretory cells as the gland assumes a quiescent state. The pre-parturient increase in tissue volume resulting from both hyperplasia and hypertrophy is accompanied by an increase in cellular differentiation as cells prepare to synthesize the principal milk constituents. This process commences some time before week 7 pre-partum and increases continuously through to peak lactation (Knight and Wilde, 1993).

Cellular differentiation is associated with an increase in the expression of key enzymes for galactopoiesis such as acetyl–CoA carboxylase and fatty acid synthetase as well as the induction of specific mRNA species encoding for the milk proteins (Wilde et *al.* 1996). The important observations of Wilde *et al.* (1987)thatthricedailymilkingincreasedboththenumber

and extent of differentiation of secretory ceils when compared with the contralateral gland milked twice per day provided strong evidence for the presence of a mammary-specific molecule that regulates the rate of cell division and differentiation and which is either secreted or inhibited by the process of milk withdrawal from the milk cistern. While these observations provide credibility for the pioneering observations of Professor Boutflour on pre-calving manipulation of the gland cited above, they do not explain the mechanisms for the induction of the genes for the key galactopoietic enzymes and milk proteins up to 7 weeks before calving.

Why determine the endocrine factors that regulate milk protein gene expression?

The commercial importance of the caseins and whey proteins for manufacturing purposes and for therapeutic uses such as the prevention of certain cancers (see Wynn et *al.* 1995) dictates the need to improve our understanding of the factors that regulate their expression at the genetic level. Clearly it is important to know more about the control of expression of a lactalbumin, for example, in view of its importance in the regulation of the synthesis of lactose, the principal osmolar determinant of milk volume. Elucidation of these mechanisms will also have implications for animal breeding and for the development of commercial transgenic animals in which the regulatory elements for the milk protein genes are used as constructs for the mammary-specific expression of high value transgenic products. The complexity of the tertiary structure of the caseins has ensured that the mammary epithelium is well endowed with the cellular mechanisms required for the synthesis of highly folded proteins dependent on their tertiary structure for the expression of full biological activity. Their expression in high quantities in milk provides a convenient vehicle from which these novel products can be readily extracted.

The methodology for the study of milk protein gene expression

Most studies which have provided this information have utilised explants dissected from tissue collected when the animal is slaughtered. This procedure is readily conducted in rodents and other small species, but is more difficult in the commercially important large ruminant species because of the complexity of the surgery and the cost. Although the use of tissue obtained from abattoirs is convenient, it is not possible to determine the physiological state of the animals at slaughter and therefore it is difficult to generate repeatable experiments. Unfortunately for the lactational biochemist, no epithelial cell lines have yet been identified from a bovine source that express the principal milk protein genes, although the Mac T line reported by Hung et *al.* (1991) did initially show promise. The refinement of gene transfection techniques will doubtless provide this essential research tool in the coming years.

Mammary biopsy is the current method of choice since the physiology of the animal can be controlled (Knight et *al.* 1992; Farr et *al.* 1994), however the published methods have met with variable success. The major implements used for the recovery of tissue have included biopsy needles and various surgical implements, the use of which causes recurring problems of excessive bleeding and oedema around the wound. Often insufficient tissue has been obtained to conduct the appropriate experiments while a further complicating factor is provided by the need to anaesthetise the animal either locally or systemically.

We have refined a biopsy methodology in which preparturient animals are anaesthetized locally through the administration of xylazine as an epidural analgesic to maintain the cow in a standing position during surgery. Bleeding is minimised by localising the vasculature of the gland using ultrasound and up to 1 Og of mammary tissue is removed by elliptical incision of tissue from the dorsal third of the forequarter. Following ligation of any blood vessels, the tissue, the lateral suspensory ligament and the skin are sutured and the cow returned to the herd. Calving and subsequent lactation do not appear to have been affected by the biopsy procedure. The tissue is then dissected to provide a source of explants, each of lmg with 20 being floated on siliconised lens paper in a cell culture medium (M199) in each culture well. These explants are viable for up to 10 days as determined by morphological examination and by the expression of a 'house keeping' gene associated with the metabolic integrity of the tissue such as β actin.

Approximately 1 00mg of tissue is required to yield sufficient RNA for the analysis of gene expression using Northern and slot blots. We have labelled full length complementary DNA probes for b and k casein and for β lactoglobulin using the non-radioactive chemiluminescent label digoxigenin (Boehringer) in the interest of safety and of minimising the exposure times of blots to photographic films. The probes have been provided by Dr Tony Mackinlay (Bonsing and Ma&inlay, 1987; Bonsing et *al.* 1988).

Identification of the key galactopoietic hormones.

The essential hormones required for the maintenance of casein gene expression in mammary explants from rats and mice have been shown to be a combination of prolactin (P) together with a glucocorticoid such as cortisol (F) and insulin (I) (Guyette et *al.* 1979; Prosser et *al.* 1987). In the presence of F and I, P is thought to improve the stability of the mRNA transcript for the

milk proteins, F is thought to act by decreasing the nuclear degradation of the message while the role of insulin in promoting mRNA accumulation is not known. Various substitutions for these key hormones are also able to induce casein gene expression including placental lactogen for proiactin, aldosterone and corticosterone for F and insulin-like growth factor 1 (IGF1) for I, although this latter factor is required at much higher levels (see Vonderhaar and Ziska, 1989). Interestingly these requirements are not universal as prolactin alone is able to induce maximal gene expression ***in vitro in*** a marsupial model, the Tammar wallaby (Maher and Nicholas, 1987). Many other growth factors and hormones have been used in culture and in general the increase in casein gene expression is not significantly greater than that achieved with the 3 principal hormones irrespective of the donor species for the tissue. Despite this effect, the level of expression achievable with the addition of these 3 hormones ***in vitro*** does not approach that observed in lactating tissue ***in vivo*** and hence there is scope for an improvement in our knowledge of the principal factors limiting casein gene expression. The optimal artificial induction of lactation ***in vivo*** through the injection of oestradiol and progesterone requires the addition of F (Head et *al.* 1980), although presumably both endogenous P and I also play a role in this process. Again this process does not replicate normal lactation suggesting that other physiological factors are required for lactogenesis.

It is important to delineate between the endocrine factors responsible for the allometric growth of mammary parenchyma. Prolactin, for exarnple, does not stimulate mammary tissue growth in the cow whether given locally or systemically, yet this hormone does so in the goat (see Forsyth, 1996). Placental lactogen induces marked growth of tissue in the cow. The lactogenic effect of this hormone, however, is subject to conjecture since the preparation used by Byatt and Bremel (1986) was ineffective ***in vitro*** whereas a recombinant source of the hormone stimulated blood a lactalbumin levels ***in vivo*** (Byatt et *al.* 1994). This effect may, however, be due to a hormonally induced increase in the "leakiness" of intercellular tight junctions in the mammary epithelium. Similarly somatotropin is not lactogenic per se, but its administration to both pre–pubertal and pubertal heifers promotes udder development (Knight and Wilde, 1993). In contrast, IGF1 is active in promoting both tissue growth and milk protein synthesis in the mouse, although the latter effect is only achieved by substituting for I at very high concentrations (Prosser et *al.* 1987). However this galactopoietic effect is only observed ***in vivo in*** ruminant species, while IGF 1 does not augment P-induced protein expression in bovine explant culture (Forsyth, 1996).

Our initial studies using tissue obtained from a cow 22 days *pre-partum* demonstrated that the basal expression of β and κ casein and β lactoglobulin genes was already high in the tissue and that this diminished markedly by 4 days in culture in the presence of I and F, and was not reversible with lactogenic concentrations of P added at day 4 (Figure 2: Sheehy et *al.* 1996). Similarly Gertler et *al.* (1982) and Wheeler et *al.* (1995) have failed to maintain lactogenesis and casein gene expression in bovine mammary explant and ovine mammary epithelial cells in primary culture respectively. In similar studies conducted subsequently in tissue collected over a range of times from day 8 to day 33 *pre-partum, the* level of basal casein gene expression was inversely related to the time from calving, with very low levels of expression being detectable at Day 33 *pre-partum* (Figure 3). In contrast the level of expression of the β actin gene fell markedly as calving approached, thereby demonstrating that the asymptote in the exponential increase in the growth of mammary parenchyma of the gland pre-partum was approaching.

Significance to the dairy producer

Thus there appears to be a definitive time prior to parturition after which the molecular regulatory mechanisms for milk protein gene expression are no longer inducible ***in vitro.*** The nature of these key regulatory elements is not well understood, although it is clear that both mammary gland specific and ubiquitous nuclear factors are involved in this process. Furthermore there appear to be negatively acting factors that influence the expression of these genes, while other regulatory elements have been located up to several kilobases upstream from the transcription initiation start site (see Groenen and van Poel, 1994). In terms of the management of the dairy cow the importance of this finding is difficult to reconcile. However it is likely that

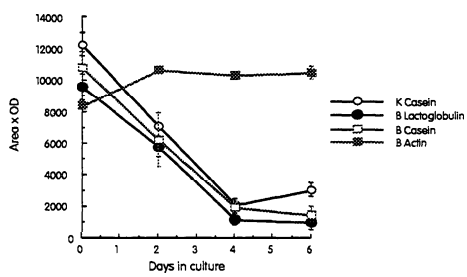

Figure 2 Milk protein gene expression in mammary

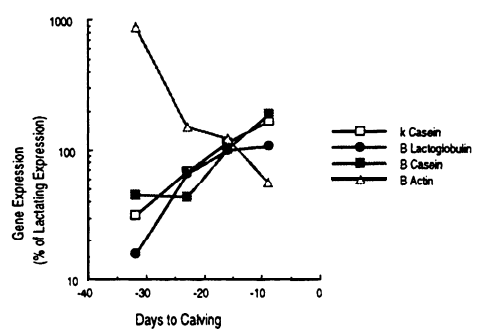

Figure 3 The influence of time pre-partum on basal milk protein geneexpression.

this period of genetic pleiotropy for casein gene expression may be an important window during which careful management of the cow is required to ensure that casein synthesis is maximised for the duration of the subsequent lactation. It is possible that the potential ceiling for casein expression is established during this early period prior to calving. Thus the identification of factors that regulate gene expression at this time will be of great significance to the dairy producer. There is little doubt that the nutritional status of the cow is likely to be an important determinant of this expression.

Also of practical importance is the observation that the length of the dry' period between lactations did not seem to alter the responsiveness of tissue ***in vitro.***

Inhibitors of milk protein gene expression: the stress hormones

In subsequent studies the influence of possible inhibitors of casein gene expression have been investigated. The role of one class of hormonal peptides which exert an interesting and diverse range of biological functions in most tissues, the opioids, has been assessed. These functions include analgesia, respiratory depression and euphoria as well as stimulating feed intake and cardiovascular activity and altering the secretory patterns of a range of hormones of pituitary, pancreatic, thyroid and gonadal origin. β endorphin is one such peptide that is released from the anterior pituitary following the enzymatic processing of the large precursor molecule proopiomelanocortin. This peptide has been identified in milk (Ferando et *al.* 1990) at low concentrations while other opioid molecules such as morphine are thought to be derived from plants ingested by animals (Hazum et *al.* 198 1). Similarly opioid peptide sequences that show similar activity to that of morphine, the casomorphins, reside within β casein, although the physiological significance of these are unknown as they are unable to produce opioid-like effects when injected systemically (Reid et *al.* 1994). In summarising these effects, it is clear that these peptides are released as part of the endocrine cascade induced in response to stress that enable the animal to adjust its metabolism to survive. Their function in milk is to modify the behaviour and metabolism of the calf thereby extending the maternal influence on the wellbeing of the neonate.

The inclusion of β endorphin in the mammary explant culture model at concentrations that are similar to normal physiological circulating levels resulted in a marked decrease in the expression of the β and κ casein and β lactoglobulin genes induced by the combination of I, F and P (Figure 5). The most remarkable observation was that at day 6 of culture the most effective dose was 50ng/mL, which suggests that this tissue is highly sensitive to the antagonistic actions of this opioid molecule. The logical implication for the management of the late pregnant cow is that any perceived stressor late in lactation may limit the induction of casein gene expression. While these observations ***in vitro*** suggest a local action transduced through the μ class of opioid receptors localised on the mammary epithelium, it is likely that these peptides also exert their inhibitory effects by manipulating the key lactogenic hormones I, F and P or their receptors.

For example β endorphin is an extremely potent inhibitor of insulin secretion from pancreatic islets at physiological concentrations (Schleicher ***et al.*** 1989), while its effects at the level of the insulin receptor/ signal transduction level in mammary tissue are unknown. In contrast, opioid agonists stimulate prolactin secretion through their ability to block the activity of the dopaminergic pathways in the hypothalamus which inhibit prolactin release (Gudelsky and Porter, 1979). However when the entire stress axis is activated through the imposition of a stressor as innocuous as milking in an unfamiliar environment, circulating prolactin levels are significantly suppressed (Bruckmaier et *al*, 1993). This decrease corresponded with a simultaneous rise in circulating cortisol and β endorphin levels. Within this context, the sensitivity of the hypothalamic–pituitary-adrenal axis is suppressed throughout

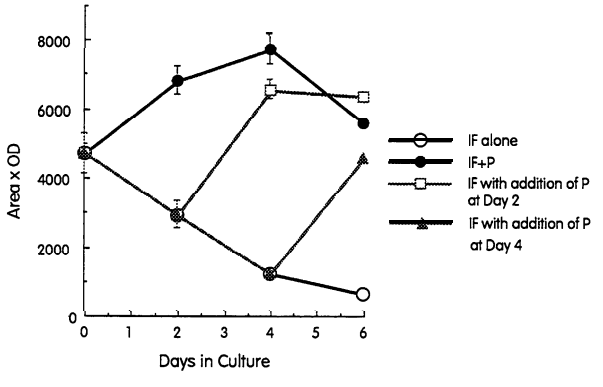

Figure 4 The induction of κ casein gene expression in tissue biopsied greater than *30* days ***pre–partum.***

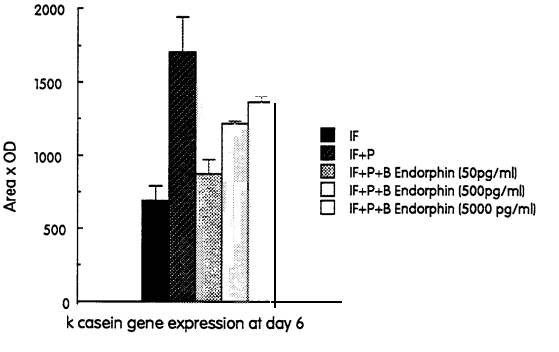

Figure 5 The influence of β endorphin on κ casein gene expression in tissue biopsied at greater than 30 days ***pre–partum.***

lactation presumably as a conservational mechanism for the nutrient supply for the calf (Lightman and Scott Young, 1989). However, little is known of the sensitivity of this endocrine axis *pre-partum,* although a peak of secretory activity occurs at the time of calving to facilitate the birth process (Patel *et al.* 1996).

The relationship between the chronic nutritional status of the animal and the sensitivity of the stress hormone axis to activation is not well documented. In fact in many stress physiology studies, the nutritional status of the animals is not considered to be an important variable. Yet the classical catabolic effects of the glucocorticoids and catecholamines in skeletal muscle tissue are well documented (Baxter and Rousseau 1979; Munck *et al*. 1984; Coderre *et al.* 1992), while hypoglycaemia is a potent stimulus for cortisol secretion in all species including ruminants (Engler *et al.* 1988). Yet many dairy producers pay little attention to the nutritional status of their pregnant cows until just before calving. The results presented here suggest that the nutritional status of the cow up to 7 weeks prior to calving may be critical to maximising lactational performance. Thus the results provide further credibility for the practice of 'steaming up' cows provided the period of supplementary feeding is extended.

Other possible inhibitors of milk protein gene expression

The family of peptides and proteins that have been demonstrated to inhibit various aspects of lactation is growing rapidly. Many of these factors are synthesised, secreted and act locally in the mammary epithelium providing exquisitely sensititive and complex regulatory mechanisms for milk secretion. The synthesis of these factors is likely to be regulated by neural or endocrine inputs under central control, although the nature of these interactions remains to be elucidated.

Of these, the most interesting is the feedback inhibitor of lactation (FIL) identified recently by Wilde and colleagues (see Wilde *et al.* 1996). The discovery of this small milk protein of M_r 6000–8000 depending on species was the culmination of 30 years of physiological experiments. The perceptive initial observations that milk secretion was dependent on the efficiency and frequency of milk removal (Linzell and Peaker, 1971) rather than the impact of milk removal on gland distention, suggested the presence of such a regulatory molecule. The unique hydrophobic nature of this complex glycoprotein delayed substantially its isolation and characterization. Experiments conducted in the goat have demonstrated that this protein inhibited both casein and lactose synthesis in explant culture and when injected directly into the teat canal, it inhibited milk secretion temporarily in a dose dependent manner (Wilde *et al.* 1995). The protein appears to act by altering the structural integrity of the golgi and endoplasmic reticulum so as to disrupt intracellular protein transport and lipid synthesis (Rennison *et al.* 1993). The acute response times to FIL, irrespective of the nature of the bioassay, suggests that the daily rhythm of milking and milk synthesis is associated with a diurnal rhythm of FIL synthesis, suggesting that more frequent and complete milking will yield more milk per day. However the need to synthesize this protein independently of the milk proteins suggests that FIL is synthesized in an inactive form and that it is activated upon secretion from the cell (see Wilde *et al.* 1996). FIL appears to have specific receptors in the mammary epitheliurn and its actions are specific for the mammary epitheliutn since it does not influence the secretory function of other secretory cell types. A possible role for this protein during the induction of milk protein gene expression *pre-partum* has not been explored, although it is possible that it plays a role in the involution of the gland and in the associated acceleration in secretory cell deletion through apoptosis.

The evidence for a locally produced mitogen being responsible for mammary tissue development has already been alluded to. It is likely that more than one of epidermal growth factor, the IGF's, aend transforming growth factor β play a role in this process in concert with putative inhibitory factors such as the mammary–derived growth inhibitor identified by Bohmer *et al.* (1987). However their role in the initiation of milk protein gene expression is largely unexplored.

The role of nutritional status in the initiation of milk protein synthesis

One factor that has not been explored in the present paper is the role of the transporter systems for rate limiting nutrients at the time of the induction of milk protein gene expression. The expression and trafficking of both the families of amino acid and glucose transporters are known to be under active endocrine control. Time course studies of their expression relative to that of the milk proteins will determine their relative importance in the initiation of lactogenesis.

There seems little doubt that the nutritional status of the pre-parturient cow will be important in determining the productivity of the subsequent lactation. The evidence presented above shows that the regulatory mechanisms for its initiation is complex, however virtually no studies have investigated the contribution of homeorhetic adjustment of the gland through the provision of adequate nutrition to the efficiency of this process. These studies will be particularly important in developing ways of minimising the impact of autocrine inhibitors of milk protein synthesis. The methodology for tissue biopsy outlined above provides a means of combining nutritional studies with the regulation of milk protein gene expression.

Strategic research of this nature will lead to a better understanding of the mechanisms restricting milk protein secretion and provide the information required to develop technologies to overcome this limitation.

References

Bauman, D.E., Vernon, R.G. (1993). Effect of exogenous prolactin administration on lactational performance of dairy *cows. Domestic Animal Endocrinology 4, 279-290.*

Baumrucker. C. R. Blum. J. R. (1993). Secretion of insulin like growth factors in milk and their effect on the neonate. *Livestock Production Science* **35**, 49–72.

Baxter, J.D. and Rousseau, G.G. (1979). Glucocorticoid hormone action: *an* overview. In: *Glucocorticoid Hormone Action* (Eds. J.D. Baxter and G.G. Rousseau). Springer: Berlin.

Bohmer, ED., Kraft, R., Otto, A., Wemstedt, C., Hellmen, U., Kurtz, A., Muller, T., Rohde, K., Etzold, G., Lehmann, W., Langen, P., Heldin, C.H. and Grosse, R. (1987). Identification of a polypeptide growth inhibitor from bovine mammary gland. Sequence homology to fatty-acid and retinoid-binding proteins. *Journal of Biological Chemistry* **262**, 15137–15143.

Bonsing, J., Ring, J.M., Stewart, A.F. and Mackinlay. A. G. (1988). Complete nucleotide sequence of the bovine β casein gene. *Australian Journal of Biological Science* **41**, 527–537.

Bonsing, J. and Mackinlay, A. G. (1987). Recent studies on nucleotide sequences encoding the caseins. *Journal of Dairy Research* **54**, 447–46 1.

Boutflour. R. (1967). *The high yielding daiy cow.* Crosby Lockwood: London.

Bruckmaier, R. M., Schams, D. and Blum, J. W. (1993). Milk removal in familiar and unfamiliar surroundings: concentrations of oxytocin, prolactin, cortisol and β endorphin. *Journal of Dairy Research* **60**, 449–456.

Byatt, J.L. and Bremel, R.D. (1986). Lactogenic effect of bovine placental lactogen on pregnant rabbit but not pregnant heifer mammary gland explants. *Journal of Dairy Science* **69**, 2066–2071.

Byatt, J.L., Eppard, P. J., Veenhuizen, J. J., Curren, T.L., Curran, D.F., McGrath, M.F. and Collier, R.J. (1994). Stimulation of mammogenesis and lactogenesis by recombinant bovine placental lactogen in steroid primed dairy heifers. *Journal of Endocrinology* **140**, 33–43.

Coderre, L., Srivastava, A.K. and Chiasson, J.L. (1992), Effect of hypercortism on regulation of skeletal muscle glycogen metabolism by epinephrine. *American Journal of Physiology 262,* E434–E439.

Delouis. C. Houdebine. L. M. Djiane. J. (1980). Relationship between hormones and mammary gland function. *Journal of Dai y Science* **63**, 1492–1 5 13.

De Peters, E. J. and Cant, J.P. (1992). Nutritional Factors influencing the nitrogen composition of bovine milk: A review. Journal of Dairy Science **75**, 2045–2070.

Engler, D., Pham, T., Fullerton, M.J., Funder, J.W. and Clarke. I.J. (1988). Studies of the regulation of the hypothalamic-pituitary-adrenal axis in sheep with hypothalmic–pituitary disconnection. I. Effect of an audiovisual stimulus and insulin-induced hypoglycemia. *Neurobiology* **48**, 551–560.

Farr, V.C., Stelwagen, K., Davis, S.R. and Cate, L.R. (1994). An improved method for routine biopsy of bovine mammary tissue. *Journal of Daiy Science* **77**, Supplement 1, 117.

Ferrando, T., Rerinero, I., De Gennaso, T., Oggero, R., Mostert, M., Dattola, P. and Pinessi. L. (1990). β endorphin like and a MSH like immunoreactivities in human milk. *Life Sciences* **47**, 633–63 5.

Forsyth, I. A. (1996) The insulin-like growth factor and epidermal growth factor families in mammary cell growth in ruminants: action and interaction with hormones. *Journal of Dai y Science* **79**, 1085–1096.

Groenen, M. A. M and Van Der Poel. J. J. (1994). Regulation of expression of milk protein genes: A review. *Livestock Production Science* **38**, 6 l-78.

Gertler, A., Weil, A. and Cohen, N. (1982). Hormonal control of casein synthesis in organ culture of the bovine lacating mammary gland. *Journal of Dairy Research* **49**, 387–398.

Gudelsky, GA. and Porter, J.C. (1979). Morphine and opioid peptide induced inhibition of the release of dopamine from the lumberoinfundibular neurons. *Life Science* **25**, 1679–1 702.

Guyette, W.A., Matusik, R.J. and Rosen, J.M. (1979). Prolactin mediated transcriptional and post–transcriptional control of casin gene expression. *Cell* 7, 1013-1023.

Hazum, E., Sabatka, J.J., Chang, K.J., Brent., D.A., Findlay, J.W.A. and Cuatrecasas, P. (1981). *Science* **213**, 1010-1012.

Haresign, W. (1979). Body condition, milk yield and reproduction *in* cattle. In: *Recent Advances in Animal Nutrition* pp. 107-122 (Eds. W. Haresign and D. Lewis). Butterworths: London.

Head, H.H., Delouis, C., Terqui, M., Kam, G. and Djiane, J. (1980). Effects of various hormone treatments on induction of lactation *in the ewe. Journal of Animal Science* **50**, 706–7 *12.*

Hung, H.T., Robitaille, G. and Turner, J.D. (199 1). Establishment of bovine mammary epithelial cells (MAC-T): An *in vitro* model for bovine lactation. *Experimental Cell Research* **197**, 191–199.

Knighy, C.H., Teverson, R.M. and Winter, A. (1992). Biopsy of the mammary gland. *British Veterinary Journal* **148**, 129–132.

Knight. C. H. and Wilde. C. J. (1993). Mammary cell changes during pregnancy and lactation. *Livestock Production Science* **35**, 3–1 9.

Lightman. S. T. Scott Young III. W. (1989). Lactation inhibits stress-mediated secretion of corticosterone and oxytocin and hypothalamic accumulation of corticotropin-releasing factor and enkephalin messenger. *Endocrinology* **124**, 23 58-2364.

Linzell, J.L. and Peaker, M. (1971). The effects of oxytocin and milk removal on milk secretion in the goat. *Journal of Physiology* **216**, 717–734.

Maher, F. and Nicholas. K.R. (1987). Pituitary induced lactation in mammary gland explants from the pregnant Tammar (*Macropus eugenii*): a negative role for cAMP. *Comparative Biochemistry and Physiology.* **87,** 1107–1 117.

Metcalf, J.A., Beever, D.E., Sutton, J.D., Wray–Cohen, D., Evans, J., Humphries, D.J., Backwell, F.R.C., Bequette, B.J. and MacRae, J.C. (1992). The effect of supplementary protein on *in vivo* metabolism of the mammary gland in lactating dairy *cows. Journal of Dairy Science* **77,** 1816–1827.

Munck, A., Goyre, P.M. and Holbrook, N.J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Reviews* **5,** 25–44.

Patel, O.V., Takahashi, T., Takahashi. W., Hirako, M., Sasaki, W. and Domeki, I. (1980). Peripheral cortisol levels througout gestation in the cow : effect of stage of gestation and foetal number. *British Veterinary Journal* **152,** 425–432.

Prosser. C. G. Samkarin. L., Hennighausen. L., Topper. Y. J. (1987). Comparison of the roles of insulin and insulin-like growth factor I in casein gene expression and in the development of a lactalbumin and glucose transport activities in the mouse mammary epithelial cell. *Endocrinology* **120,** 1411–1416.

Prosser, C.G., Fleet, I.R., Corps, R.N., Froesch, E.R. and Heap, R.B. (1990). Increase in milk secretion and mammary blood flow by intra-arterial infusion of insulin-like growth factor-I into the mammary gland of the goat. *Journal of Endocrinology* **126,** 437–443.

Reid, L. D. and Hubbel. C. L. (1994). An assessment of the addiction potential of the opioid associated with milk. *Journal of Dai y Science* **77,** 672–675.

Rennison, M.E., Kerr, M.A., Addey, C.V.P., Handel, S.E., Turner, M.D., Wilde, C.J. and Burgoyne, R.D. (1993). Inhibition of constitutive protein secretion from lactating mammary epithelial cells by FIL (feedback inhibitor of lactation), a secreted milk protein. *Journal of Cell Science* **106,** 641–648.

Schleicher, R.L. (1989). β endorphin inhibits insulin secretion from isolated panceatic islets. *Endocrinology* **124,** 1254–1258.

Sheehy, P.A., Nicholas, K.R. and Wynn, P.C. (1996). Maintenance of bovine milk protein gene expression *in vitro. Proceedings of the Australian Society of Animal Production* **21,462.**

Sutton, J. (1992). *Proceedings of the University of Sydney Dairy Research Foundation Symposium,* **1-7.**

Vonderhaar, B.K. and Ziska, S.E. (1989). Hormonal regulation of milk protein gene expression. *Annual Review of Physiology* **51,** 641–652.

Wilde, C.J., Henderson, A.J., Knight, C.H., Blatchford, D.R., Faulkner, A. and Vernon, RJ. (1987). Effects of long term thrice daily milking on mammary enzyme activity, cell proliferation and milk yield. *Journal of Animal Science* **64.** 533–539.

Wilde, C.J., Addey, C.V.P., Boddy, L.M. and Peaker, M. (1995). Autocrine regulation of milk secretion by a protein *in* milk. *Biochemical Journal* **305,** 5 l-58.

Wilde, C. J., Knight, C.H. and Peaker, M. (1996). Autocrine regulation of milk secretion. In: *Progress in Dairy Science* (Ed. C.J.C. Phillips). CAB International: London.

Wheeler, T.T. and Wilkins, R.J. (1995).Milk protein synthesis, gene expression, and hormonal responsiveness in primary cultures of mammary cells from lactating sheep. *Experimental Cell Research* **217, 346-354.**

Wynn, P.C., Sheehy, P.A., Bolouri–Oskoui, K., Behrendt, R. and Nicholas, K.R. (1995). Making more out of milk: a challenge to the biotechnologist. *Proceedings of the Nutrition Society of Australia* **19,** 80–87.