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**Modification of Milk Composition via Transgenesis:
The Role of extracellular Matrix in Regulating
Transgene Expression**

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Abstract

Altering milk composition via transgenesis depends on three main factors. (1) The availability of an efficient regulatory sequences for targeting transgene(s) to the mammary gland; (2) a reliable in vitro model to test the expression of transgenes prior to their introduction to the animal genome; and (3) better understanding of the major factors which determine the rate of gene expression and protein synthesis. The current studies provide the necessary means and knowledge to alter milk protein composition via transgenesis. The following specific goals were achieved:

a: Identifying regulatory regions in the β -lactoglobulin (BLG) gene and the cross-talk between elements which enabled us to construct an efficient vector for the expression of desirable cDNA's in the mammary gland.

b: The establishment of a sheep mammary cell line that serves as a model for the analysis of endogenous and exogenous milk protein synthesis in the mammary gland of livestock.

c: An accurate comparison of the potency of the 5' regulatory sequences from the BLG and whey acidic protein (WAP) promoters in directing the expression of human serum albumin (HSA) to the mammary gland in vitro and in vivo. In this study we have also shown that sequences within the coding region may determine a specific pattern of expression for the transgene, distinct from that of the native milk protein genes.

d: Characterizing the dominant role of ECM in transgene expression in mammary epithelial cells.

e: Further characterization of the BCE-1 enhancer element in the promoter of the β -casein gene as a binding site for the c/EBP- β and Stat5. Identifying its interaction with chromatin and its up regulation by inhibitors of histone deacetylation.

f: Identifying a mechanism of translational control as a mediator for the synergistic effect of insulin and prolactin on protein synthesis in the mammary gland.

Objectives of the original research proposal

1. To define specific cis-acting DNA sequences regions within the BLG gene responsible for reproducibly high level of expression.
2. To define the cellular basis for the apparent different mode of expression of transgene and endogenous milk protein genes.
3. To study cell-cell and cell-ECM interactions that are crucial for the efficient synthesis and secretion of endogenous and exogenous proteins in the mammary gland.

Relevant data, methodology, results and discussion

Milk is a basic nutrient in human and animal food. Modification of milk composition via transgenesis may lead to food of higher quality, save the cheese industry considerable resources, enable distributors to cater niche markets and perhaps aid in generating a healthier livestock. Much is still unknown about the regulation of milk protein gene expression in the mammary gland. Thus, the current project was focused on sequences required for efficient targeting of expression of heterologous proteins to the mammary gland and understanding the basic principals of hormonal and cell- extracellular matrix control of native and foreign gene expression in the gland.

The original specific aims of this research were¹:

1. To define cis-acting DNA sequences regions within the BLG gene responsible for reproducibly high level expression.
2. To define the cellular basis for the apparent different mode of expression of transgenes and endogenous milk protein genes.

¹ The abbreviation used are: BLG - β -lactoglobulin, ECM - Extracellular matrix, EHS - Engelbreth-Holm-Swarm, FCS - Fetal calf serum, HSA - Human serum albumin, MAP K - Mitogen-activated kinase, PHAS-I Phosphorylated heat- and acid-stable protein regulated by insulin, PRL- prolactin, STAT- signal transducer and transactivator of transcription, WAP - Whey acidic protein.

3. To study the cell-cell and cell-ECM interactions that are crucial for the efficient synthesis and secretion of endogenous and exogenous proteins in the mammary gland.

During these studies we also addressed the following additional aims:

1. Analysis of transcriptional mechanisms of extracellular matrix-driven tissue-specific gene expression: Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and ECM and modulated by the state of histone acetylation.
2. Hormonal regulation of native and transgenic milk protein expression.
3. Establishment of a sheep mammary epithelial cell line to serve as a model for the analysis of native and foreign milk proteins in the mammary gland.

Defining cis-acting DNA sequences regions within the BLG gene responsible for reproducibly high level expression.

The promoter of the major milk protein genes can be used to target the expression of any gene to the mammary gland of transgenic animals resulting in the production of desired proteins in the milk. Although mice and sheep carrying genes composed of genomic sequences of human $\alpha 1$ antitrypsin or HSA behind the BLG promoter secrete high level of the corresponding proteins, almost no expression was detected in the mammary gland of transgenic mice carrying the cDNA of these genes.

In the generation of transgenics, the use of vector comprised of a cDNA sequence encoding the desired protein has certain advantages over the use of vector comprised of genomic sequences. The cDNAs are usually more available than the genomic sequences and their significant shorter length makes vector construction easier. However, their poor performance in directing protein expression in transgenic mice, attributed to the increased sensitivity to chromosomal position effect and difficulties in maintaining active chromatin configuration has been problematic. The present study was undertaken to design a BLG vector that enables efficient expression of cDNAs in the mammary gland by identifying regulatory sequences and cross-talk between elements in the BLG gene with the reporter - the

luciferase cDNA. The basis for this study was a previously prepared BLG expression cassette in which cDNA's were inserted between intron1 and 6 of the BLG gene.

Here, the BLG/luciferase constructs were stably transfected to CID-9 mammary epithelial cells that served as model for the differentiating mammary gland. Analysis of luciferase expression was performed in cells cultured on laminin rich matrix (Matrigel) for 6 days in the presence of insulin hydrocortisone and prolactin. (Fig 1). Additional experiments were carried out to determine the relative contribution of each hormone to the luciferase activity.

Regulatory elements in BLG intron 1 and the role of SV 40 PA sequences.

Construct 907 is composed of the original BLG expression cassette (Barash, I., Nathan, M., Kari, R., Ilan, N, Shani, M. and Hurwitz, D.R. *Nuc. Acids. Res.* 24:602-610, 1996) containing the luciferase reporter cDNA inserted between intron 1 and 6. Comparing the expression of construct 907 vs. 914, shows that luciferase activity, when driven by elements of the BLG gene (i.e. BLG promoter, and exon 6 and 3' flanking region), depends on the presence of BLG intron 1. In its absence, there is a dramatic decline in activity.

Surprisingly, a tenfold increase in luciferase activity was detected when the BLG cassette contained sequences of the SV40 polyadenylation (PA) site, inserted downstream of BLG exon 6 (see construct 904). The luciferase activity induced by this construct served as a reference (100%) for the comparison with all other vectors. The BLG PA sequences in construct 904 were not expected to contribute to the expression of cDNA constructs since the SV40 PA sequences were placed in front of them. However their elimination (constructs 921) resulted in a 2.5 fold decline in the luciferase activity. It could be that the BLG PA sequences and 3' serve, at least in this case, as insulator sequences from the suppressing effects of host DNA sequences at the site of integration. Interestingly, the contribution of the BLG PA sequences in these constructs appears to be specific, since no further decrease in luciferase activity could be detected by eliminating the entire BLG 3' region (construct 906).

Also, it should be noted that BLG intron 1 contributes to luciferase activity even in the context of the SV40 PA site (compare construct 904 vs. 915). This contribution appears to be specific to BLG gene sequences since their substitution

Fig.1 Schematic representation of BLG/Luciferase vectors used to transfect CID-9 cells. Luciferase cDNA inserted in the first untranslated BLG exon 1, or in the second exon of the BLG gene, or behind the complete BLG gene were stably transfected into mammary epithelial cells of the CID-9 line. For determination of luciferase activity, cells were cultured on Matrigel in the presence of insulin, prolactin and hydrocortisone for 5 days. Each analysis was performed in triplicates and values were related to those obtained with construct 904. The presented values are mean of more than 3 independent assays.

with insulin intron 1 sequences did not restore luciferase activity (construct 915 vs. 918).

Regulatory elements within the distal part of the BLG promoter and their interaction with BLG intron 1.

Luciferase activity with constructs containing BLG intron 1, SV40 PA and BLG 3' declined when 1kb of the 3kb BLG promoter was deleted from its distal end (construct 920 vs. 904). However, with constructs devoid of BLG intron 1, deleting 1kb of the BLG promoter resulted in a significant increase of over 3 fold in luciferase activity (915 vs. 925), and even in the absence of BLG 3' sequences (construct 908 vs. 919). Taken together, deleting the distal 1kb of the BLG promoter in construct 925 led to a 3.9 fold higher luciferase activity in transfected CID-9 cells than with construct 904, that serves as our reference. This level of activity with construct 925 was 39 fold higher than with our original BLG cassette (construct 907).

Why deleting the distal end of the BLG promoter results in an opposite effect, depending on the presence or absence of BLG intron 1, remains to be determined. Our working hypothesis is that there is a cross-talk between elements within the distal end of the BLG promoter and within intron 1. Experiments are underway to address this hypothesis by testing a series of new gene constructs carrying various deletions in that region.

It should be noted that only marginal activity was observed when the luciferase cDNA was inserted behind the entire BLG gene (construct 916) and essentially no activity was detected with a promoterless luciferase cDNA construct (construct 910).

BLG elements and hormonal effects in transfected CID-9 cells.

We tested the responsiveness of the various BLG expression cassettes to the inductive effect of lactogenic hormones. To this end we used only the more efficient SV40 PA containing constructs that allowed better evaluations of subtle differences. Table 1 shows comparable rates of induction by hydrocortisone and prolactin.

Table 1. Hormonal effect on the expression of BLG/Luciferase constructs in CID-9 cells.

Construct	Composition				Expression (% of IFP)					
	BLG	BLG	BLG 3'	SV40	Matrigel			Plastic		
	5'	int.1		enh	I	IP	IFP	I	IP	IFP
	(kb)									
904	3	+	+	+	30	48 (18)	100 (52)	18	39 (21)	100 (61)
920	2	+	+	+	29	39 (10)	100 (61)	18	60 (42)	100 (40)
906	3	+	-	+	37	55 (18)	100 (45)	30	32 (2)	100 (68)
915	3	-	+	+	28	50 (22)	100 (50)	6	48 (42)	100 (52)
921	3	+	+/-	+				48	64 (18)	100 (36)
924	3*	+	+	+	10	26 (16)	100 (74)	41	57 (16)	100 (43)
919	2	-	-	+	61	78 (17)	100 (22)			
925	2	-	+	+	8	35 (27)	100 (65)	43	58 (15)	100 (42)

Transfected CID-9 cells were cultured for 5 days in the presence of insulin (I), prolactin (P) and hydrocortisone (F) in various combinations. Luciferase activity was determined for each construct at the termination of the culture period, and is presented as percent of that achieved in cells treated with IFP. In parenthesis - the relative contribution of prolactin and hydrocortisone to luciferase activity.

+/- lacking only the BLG PA site. * Restricted.

(over the basic activity achieved with insulin alone) with all transfected constructs, independent of the presence of BLG intron 1, or sequences of the distal promoter. This suggests that the differences in expression between the various constructs are not due to altered response to lactogenic hormones. However, while in previous studies we demonstrated major induction of casein and BLG by prolactin with a minor contribution of hydrocortisone, the expression of BLG/luciferase constructs used in this study was highly dependent of the addition of hydrocortisone whether or not cells were cultured on plastic or Matrigel.

Expression in transgenic animals (preliminary analysis):

To test whether elements in the BLG expression cassette that function in vitro can also operate in vivo, transgenic mice carrying constructs 907 and 904 were generated.

None of the 5 transgenic lines carrying construct 907 expressed detectable levels of the luciferase RNA, and marginal luciferase activity was detected in the mammary gland of only one line.

From 3 transgenic lines carrying construct 904 two expressed the luciferase RNA and one had a very high luciferase activity in the mammary gland (about 1000 fold over control level). These results suggest that the SV40 PA sequences contribute also to the in vivo activity of the BLG expression cassette to direct expression of cDNA to the mammary gland. They also suggest that stable

transfected CID-9 cells may serve as a model to test the potential of BLG based constructs to be expressed in vivo prior to their introduction into the mammalian genome.

The cell-cell and cell-ECM interactions that are crucial for the efficient synthesis and secretion of endogenous and exogenous proteins in the mammary gland: Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation (Ref. 1).

We (M.J.B) have shown that ECM regulates selectively the set of genes expressed in mammary epithelial cells. To understand the ECM signal transduction pathway leading to tissue-specific gene expression in functionally normal mammary epithelial cells, the β -casein gene, has been studied. This gene is a well known tissue-specific gene encoding for a milk protein and is used as a marker of epithelial differentiation. The first ECM-responsive element to be discovered was isolated in the 5' regulatory sequence of the β -casein gene (Schmidhauser, C., Bissell, M.J., Myers, C.A. and Casperson, G.F. *Proc. Natl. Acad. Sci.* 87:9118-9122, 1990; Schmidhauser, C., Casperson, G.F., Myers, C.A., Sanzo, K.T., Bolten, K. and Bissell, M.J. *Mol. Biol. Cell* 3:699-709, 1992). The shortest DNA sequence of this gene able to integrate the integrin-ECM signaling, was shown to be a 160bp enhancer.

The ECM-responsive element of the β -casein gene is recognized by C/EBP- β and STAT-5.

Studying the transcription factors able to bind to the ECM-responsive element, site specific mutagenesis of the ECM-responsive element has been performed. Mutations were generated across the enhancer by sequential replacement of 10bp stretches with an XbaI site flanked by GCs. In the presence of ECM and prolactin, five mutations resulted in a loss of at least 90% activity as compared to the wild type enhancer. These mutations were localized to two separate regions: a 5' region within the first 100bp which spans across a putative C/EBP- binding site and a 3' region

in the last 60bp which overlaps with a putative STAT-5 binding site. Electromobility shift analysis with oligonucleotides to the functionally important regions have been performed. Nuclear extracts contained an activity complexing with a C/EBP- β site. These complexes could be disrupted with either cold excess of this oligonucleotide or a consensus oligonucleotide to C/EBP- β . Furthermore, addition of an antibody to C/EBP- β but not to C/EBP- α resulted in a supershifted complex. These results established that the 5' region of this 160 bp enhancer is required for its function and is able to bind C/EBP- β . Similar experiments have shown that STAT-5 and an as yet unidentified factor are able to bind to the 3' region of this enhancer and are essential for its function. However, despite the strong transcriptional induction by ECM, no differences in *in vitro* binding activity to these regions were observed in extracts from cells cultured in the presence or absence of ECM.

The ECM-responsive element of the β -casein gene functions only in a chromatin context.

By transient transfection analyses we screened several enhancer-promoter constructs which when integrated into the genome were known to respond either positively or negatively to the presence of ECM. In these experiments, the ECM-responsive enhancer and the MMTV enhancer were linked to a minimal MMTV promoter. ECM did not enhance transcription on a transient template though endogenous β -casein was differentially expressed under these culture conditions as shown by immunoprecipitation of milk proteins. The requirement for stable integration of the ECM-responsive enhancer as well as the known role of chromatin reorganization in MMTV induction, led us to analyze the role of chromatin structure in ECM-induced transcription. We conducted a number of *in vivo* exonuclease footprinting analyses in collaboration with the laboratory of Dr. Gordon Hager (NCI, NIH). We examined chromatin in nuclei isolated from transfected CID-9 cells which were cultured in conditions of active or inactive transcription. To ascertain the transcriptional state of the cells, we routinely performed CAT assays on aliquot of cells taken before the nuclei were isolated. Nuclei were treated with different restriction enzymes to study either the 5' or the 3' region of the enhancer. Stops have been identified within the boundaries of C/EBP- β and STAT-5, however, no differences in the stop patterns were observed under any of the culture conditions tested. This exonuclease study indicates that the three transcription factors are constitutively loaded on their chromatin template, regardless of the ECM presence.

β -casein transcription is turned on by inhibitors of histone deacetylases.

Treatment of the mammary epithelial cell line CID-9, stably transfected with the ECM-responsive element, with sodium butyrate on plastic culture dishes induced transcriptional activity in the absence of ECM. Since sodium butyrate treatment has been shown to induce many changes in the cell in addition to inhibition of histone deacetylase, we tested a new specific inhibitor of histone deacetylase, trichostatin A. Trichostatin A is able to bind directly to histone deacetylase enzymes and works at nanomolar concentrations. A dose-dependent induction of transcription was also observed with trichostatin A in cells cultured in the absence of ECM. As a control of specificity, we have shown that the transcription of a MMTV construct stably-transfected in CID-9 cells was shut down.

Perspectives.

We are now in a position to ask what is the mechanism by which ECM modulates chromatin structure, leading to gene activation and differentiation in our model system?

The results mentioned above indicate that the ECM-responsive element can be activated without ECM, if the ECM signaling is bypassed at the histone level. We have now shown that histone H4 hyperacetylation precedes and correlates with the ECM-responsive element activation, using specific antibodies directed against acetylated lysines (Pujuguet et al., in preparation). We are also investigating the possibility that ECM itself regulates the enzymes regulating the histone acetylation status. Preliminary results indicates that the intranuclear level of HDAC1, the only known enzyme able to deacetylate histone, is downregulated in differentiating cells. Furthermore, transfection of HDAC1 in functionally normal mammary epithelial cells abrogates their ability to differentiate in the presence of ECM (Pujuguet et al., in preparation).

Regulation of transgene expression by cell - extracellular matrix interactions: The BLG/HSA fusion genes do not respond accurately to signals from the extracellular matrix in mammary epithelial cells from transgenic mice (Ref. 2).

Previously, we demonstrated that non-HSA -secreting transgenic strains retain the potential to express the HSA transgene in vitro and that mammary epithelial cell

cultures from non-HSA secreting strains express higher levels of HSA when grown on tissue culture plastic than they do when grown on collagen. In this study, we studied the expression of BLG/HSA fusion genes in epithelial cell cultures of additional transgenic strains and additional substrata. Our results show that: (1) the BLG/HSA fusion gene in only one of seven HSA-secreting or nonsecreting transgenic strains tested, responded to signals from the EHS matrix. (2) HSA DNA sequences dominantly affected the activity of BLG as well as the whey acidic protein (WAP) promoters and (3) HGF/SF induced both milk proteins and HSA gene expression.

These results suggest that the response to the ECM plays a key role in the expression of BLG/HSA fusion genes and that the function of the regulatory elements within the promoter regions of milk protein genes, involved in response to the ECM, in developmental and in tissue specificity, very much depend on the downstream gene sequences.

Hormonal regulation of native and transgene expression (Ref. 3,4).

Activation of milk protein genes in mammary cultures depends on the synergistic effect of lactogenic hormones. This dependency has also been established in the activation of transgenic proteins encoded by hybrid genes composed of native milk protein promoter and the coding sequences from another source. While differences were reported in the requirement of hydrocortisone and prolactin for the activation of WAP and β -casein genes in rat mammary organ cultures, insulin is considered the basic prerequisite for the induction of milk proteins in rodent (except for rabbits) and bovine cultures.

Although the metabolic pathways of insulin and prolactin are under intensive investigation, their synergistic role in stimulating milk protein expression remained an enigma. We have previously generated lines of transgenic mice in which the expression of the HSA gene or minigene is directed to the mammary gland by the sheep BLG promoter. The hybrid BLG/HSA gene switch on their responsiveness to lactogenic hormones during development and a similar pattern of responsiveness to insulin hydrocortisone and prolactin was seen in organ culture. The exceptional feature of BLG/HSA gene expression compared to the endogenous milk proteins is

its relatively high level of expression in the mammary gland of virgin mice. This early developmental expression enabled us to distinguish between transcriptional and post-transcriptional regulation during early pregnancy.

In mammary organ cultures of transgenic mice carrying several BLG/HSA transgenes we have observed, in this project, a dramatic uncoupling of the expression of the HSA RNA and protein. Upon explantation, both HSA RNA and protein were expressed at high levels. However when the explants were grown in hormone-free medium, HSA RNA continued to accumulate, whereas the synthesis of the corresponding protein was dependent on the presence of insulin and prolactin with minor contribution of hydrocortisone. The untranslated HSA RNA was indistinguishable from its translatable counterpart in its mobility on agarose gels, was transported normally from the nucleolus to the cytoplasm and was translated efficiently in rabbit reticulocyte lysate. In the presence of cycloheximide, an inhibitor of protein synthesis, HSA RNA rapidly disappeared, suggesting a dependency on ongoing protein synthesis. Its estimated half-life of 5-6 h in hormone free medium increased significantly in the presence of insulin hydrocortisone and prolactin and was comparable to that of β -casein RNA.

The uncoupling of the expression of the BLG/HSA transgenes at the RNA and protein levels was also confirmed by in situ hybridization and immunohistochemistry of sections from virgin mammary explants. HSA synthesis was initiated within 13 h of the addition of insulin and prolactin in explants that had accumulated untranslated HSA RNA and was fourfold higher than that observed with insulin alone. Addition of hydrocortisone contributed to an additional 20% in HSA synthesis. Interestingly, HSA translation response is much quicker than the onset of β -casein transcription mediated via the binding of MGF (Stat5a) to promoter sequences in HC-11 cells or in COS cells transfected with cDNA of MGF and PRL-receptor. We believe that this was the first demonstration of translational control of exogenous milk protein gene expression in the mammary gland of transgenic animals.

Prolactin and insulin synergize to regulate the translation Modulator PHAS-I via mitogen-activated protein kinase-independent but Wortmannin- and Rapamycin-sensitive pathway.

To probe further into the synergistic effect of insulin and prolactin on protein synthesis in the mammary gland we have initiated a study with differentiating CID-9 mammary epithelial cell line.

It has been shown that the JAK2/Stat5 pathway mediates PRL transcriptional signal transduction leading to the initiation of milk protein synthesis. The binding of STAT5 to specific motifs in the promoters of caseins, BLG and WAP genes promote gene transcription. However, it has been shown in the HC11 mammary cell line that PRL activation of Stat5/MGF is insulin independent, whereas the expression of the β -casein gene depends on the presence of insulin and hydrocortisone.

In addition to the JAK2/Stat5 pathway, PRL stimulation of cellular function has been demonstrated to involve several other Tyr and Ser/Thr phosphorylation pathways including those catalyzed by MAP kinase, S6 kinase, Raf-1, protein kinase C phosphoinositol (PI) 3'-kinase and fyn. However no conclusive evidence for synergism with insulin could be drawn from these studies dissecting these pathways.

Recently, it has been shown that insulin initiates protein translation activity in NIH 3T3 cells and fat tissue by inducing the phosphorylation of PHAS-I, a binding protein to the translation initiation factor eIF-4E. The phosphorylation of PHAS-I, results in its dissociation from the eIF-4E, enables binding of eIF-4G and allow the formation of an active complex which induce the initiation of translation by augmenting mRNA cap binding ability to binding the small ribosomal subunit.

Our working hypothesis was that the synergism between insulin and prolactin on protein production in the mammary gland may be mediated via the regulation of protein translation. This hypothesis was studied in differentiating mammary epithelial CID-9 cells cultured on laminin-rich matrix and treated with insulin alone for 5 days. Prolactin was added to the insulin treated cells on the 6th day for 0 -180 min.

We demonstrated that the phosphorylation of PHAS-I depends on the presence of both Insulin and prolactin in the culture medium and resulted in the dissociation of PHAS-I from the eIF-4E translation initiation factor. This step is crucial for the initiation of translation. The induction of PHAS-I phosphorylation was rapid and its rate matched that demonstrated for the JAK2/STAT5a and the binding of STAT5a to its DNA binding motif. However, 120 min were needed for complete phosphorylation of PHAS-I, evidenced by the shift of the protein to its highly phosphorylated, slower migrating γ form.

In the presence of insulin, PRL induced MAP kinase activity at a comparable time course to that of PHAS-I phosphorylation. However, a line of evidence suggested that although this kinase phosphorylates PHAS-I *in vitro*, it does not actively participate in its phosphorylation *in vivo*: (a) the level of insulin needed to enable PRL-induced ERK-1/ERK-2 activation was one order of magnitude higher than that needed for PHAS-I phosphorylation and (b) PD 098059, a MEK-1 inhibitor, completely inhibited insulin-dependent, PRL-induced ERK-1/ERK-2 activation but had no effect on PRL-induced PHAS-I phosphorylation. In contrast, wortmannin, a PI 3' kinase inhibitor, and the immunosuppressant rapamycin abrogated PHAS-I phosphorylation and caused a reciprocal shift between the fully phosphorylated PHAS-I γ form and its non-phosphorylated α form. We speculate that the synergism between insulin and PRL in activation of PHAS-I phosphorylation is mediated via their effects on the IRS1 and/or PI 3' kinase. Since the partly phosphorylated PHAS-I β form was not significantly affected by these inhibitors, it is possible that this synergistic effect is generated by two kinases.

In vivo and in vitro expression of HSA genomic sequences in mammary epithelial cells with BLG and WAP promoters The apparent different mode of expression of transgenes and endogenous milk proteins (Ref. 5).

The control elements of the major milk protein genes are being exploited to target the expression of foreign genes to the mammary gland of transgenic animals. In search for *in vitro* models for the efficiency of transgene expression in the mammary gland, we have compared the *in vivo* and *in vitro* expression of HSA genomic sequences in mammary epithelial cells with the BLG and WAP promoters. We also characterized the apparent heterogeneous pattern of the HSA transgene expression observed in BLG/HSA transgenics and compared it with the *in vivo* pattern of expression of same HSA genomic sequences driven by the rat WAP promoter. Previously, we have demonstrated heterogeneous pattern of expression of the BLG/HSA genomic sequences in the mammary gland of transgenic mice as opposed to the homogenous pattern of expression of the native milk protein genes.

The expression pattern of HSA in transgenic mice carrying various HSA genomic sequences driven either by the mouse WAP or the sheep BLG promoters, was compared. The pattern of HSA expression in both WAP/HSA and BLG/HSA transgenic lines was copy number independent, and the major site of ectopic expression was the skeletal muscle. Although an equal proportion of expressors was determined in both sets of mice (~25% secreting >0.1 mg/ml), the highest level of HSA secreted into the milk in the WAP/HSA transgenic lines was one order of magnitude lower than in the BLG/HSA lines. Despite this difference, the HSA expression patterns in the mammary gland was similar and consisted of two levels of variegated expression, correlated with the number of copies of the transgene. Studies using mammary explant cultures revealed a comparable responsiveness to the lactogenic hormones insulin, hydrocortisone and prolactin, although the WAP/HSA gene constructs were more sensitive to the hydrocortisone effect than were the BLG/HSA vectors. When HSA vectors were stably transfected into the mouse mammary cell line CID-9, they displayed a hierarchy of expression, dependent upon the specific complement of HSA introns included. Nevertheless, the expression of HSA in four out of five WAP/HSA constructs was similar to their BLG/HSA counterparts. This construct-dependent, and promoter-independent, hierarchy was also found following transfection into the newly established GOLDA-1 ovine mammary epithelial cell line. With the increasing importance of culture models to test gene constructs prior to the formation of transgenic animals, the in vitro/in vivo comparisons of the WAP/HSA and the BLG/HSA gene expression were informative. While the proportion of expressors were the same, the 10-fold difference in expression in vivo was eliminated in culture. Furthermore, transfection of CID-9 and GOLDA-1 cells with the various HSA vectors may provide further information about putative regulatory elements within the HSA gene sequences.

Establishment of a sheep mammary epithelial cell line to serve as a model for the analysis of endogenous and exogenous milk protein synthesis in the mammary gland. Establishment and initial characterization of the ovine mammary epithelial cell lines NISH and GOLDA-1 (Ref. 6).

Many tissue culture models (organ cultures, primary cultures, mammary epithelial cells and cell lines) have been established to better understand the complex biology of the mammary gland. For years, the only functional and well-characterized cell line available was the COMMA-1D cell line, derived from normal pregnant mice. The COMMA-1D and its subclones HC-11 and CID-9 cell lines were used extensively to study the effect of lactogenic hormones and ECM on milk protein gene expression. Although, many other mammary epithelial cell lines have been established from human and rodents and contributed to our knowledge of mammary gland physiology, very few cell lines are available from the major milk protein producers, the farm animals. The establishment and study of mammary epithelial cell lines from farm animals is of special interest because the rodent model does not accurately reflect udder development and physiology. No mammary cell lines have been established from sheep or goat, and only four bovine cell lines are currently available. None of these cell lines show both hormonal and ECM response and only one of them (BME UV) shows marginal casein synthesis. These cell lines are therefore unsuitable for the study of the effect either of the hormonal regulation of ovine/bovine casein expression or the effect of ECM on milk protein gene expression.

In the current study we established an heterogeneous functional epithelial cell line (NISH) and its sub-clone GOLDA-1 which may represent the stem cells of the mammary gland. When plated in or on top of the appropriate ECM, both NISH and GOLDA-1 cells secrete BLG (Golda -1 was analyzed also for β -casein synthesis and found positive) in an ECM dependent manner, and are capable of forming structures resembling ducts, lateral buds and alveoli. Studies were completed for NISH cells and are currently continued for the GOLDA-1 clone.

Interestingly, the presence of growth hormone dramatically increased BLG secretion from NISH cells cultured on ECM. It appears that GH is required not only to establish the structural organization, but is also continuously needed to maintain BLG expression in these cells. Stable transfection of NISH cells with BLG /HSA gene constructs revealed that the relative level of expression was comparable to the *in vivo* secretion of HSA in transgenic mice carrying these gene sequences. No transcripts could be detected in cells transfected with hybrid genes carrying either

HSA cDNA or the entire HSA gene, and HSA expression was dependent on the presence of intronic sequences.

Some minor differences were observed between the heterogenous population of NISH cells and its sub-clone GOLDA-1 which demonstrated the same morphology as shown for the NISH when cultured on matrix, but was less dependent on GH for organization and milk protein synthesis. Golda 1 cells were also successfully transfected with both BLG/HSA and WAP/HSA gene constructs and displayed an hierarchy of expression, dependent upon specific complement of HSA introns included.

These results demonstrate that NISH and GOLDA-1 cells may provide useful tools for studying the differentiation and organization of mammary epithelial cells under defined culture conditions. Furthermore, transfection of these cells may be an alternative for the transgenic mouse model in evaluating the potential of gene constructs to be efficiently expressed in the mammary gland of transgenic farm animals.

Evaluation of the research achievements as related to the original research proposal

All the aims of the original research proposal were addressed. We have made progress in identifying regulatory sequences in the BLG gene and pointed towards a possibility of cross-talk between those elements. The dominant role of the ECM in regulating transgene expression was investigated and the special in situ pattern of transgene expression in the tissue was demonstrated.

In addition, we have made progress in the study of three related subjects that were not part of the original proposal: (1) Analysis of transcriptional mechanisms and characterization of a transcriptional enhancer in the promoter of the β -casein gene. (2) Hormonal regulation of endogenous and transgene expression in the mammary gland with a focus on a translational regulation. (3) Establishment of a cell line from the mammary gland of a farm animal to serve as a model for studying milk protein gene expression in the gland of ruminants. Overall, the results of these studies were published in 6 manuscripts.

Description of cooperation

The US group cloned and characterized the CID-9 mammary epithelial cells and made them available to the Israeli group. Here, these cells served as a model to analyze the feasibility of transgene expression prior to its introduction to the host genome and the production of transgenic animals. The US group identified the BCE-1 element in the promoter of the β -casein gene and shared the knowledge and techniques with the Israeli group who focused on the BLG gene. Together, we have published one manuscript (#5 in the references list) and share the unpublished results concerning identifying elements in the BLG gene.

Conclusions

Altering milk composition via transgenesis is now feasible for any cDNA using the BLG expression cassette. The pattern and the level of transgene expression is determined by the regulatory sequences derived from the milk protein gene, but is also affected by sequences within the region, the structure of the chromatin at the site of integration and the interactions between the epithelial cells and the ECM. Indeed, the ECM which is essential for native gene expression in the gland, may also exert negative effects on transgene expression. In vitro culture systems, derived from mammary cell lines of mouse or ovine mammary gland, are valuable in evaluating regulatory sequences, both in the promoter and in the coding region. However, these models do not accurately mirror the overall pattern and feasibility of transgene expression in the gland.

Publications which resulted from the studies supported by this grant

1. Myers, C.A., Schmidhauser, C., Mellentin-Michelotti, J., Fragoso, G., Roskelley, C.D., Casperson, G., Mossi, R., Pujuguet, P., Hager, G. and Bissell, M.J. Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. ***Molecular and Cellular Biology*** 18:2184-2195, 1998

2. Ilan, N., Barash, I., Raikhenstein, M., Faerman, A. and Shani, M. (1996). β -lactoglobulin/Human serum albumin fusion genes do not respond accurately to signals from the extracellular matrix in mammary epithelial cells from transgenic mice. ***Experimental Cell Research* 228**: 146-159.
3. Baruch, A., Shani, M. and Barash, I. (1998). Insulin and prolactin synergize to induce translation of human serum albumin in the mammary gland of transgenic mice. ***Transgenic Research* 7**:15-27.
4. Barash, I. (1999). Prolactin and insulin synergize to regulate the translation Modulator PHAS-I via mitogen-activated protein kinase-independent but Wortmannin- and Rapamycin -sensitive pathway (**submitted**).
5. In vivo and in vitro expression of Human serum albumin genomic sequences in the mammary epithelial cells with β -lactoglobulin and Whey acidic protein promoters. ***Molecular Reproduction and Development* 52**:241-252.
6. Ilan, N., Barash, I., Gootwine, E. and Shani, M. (1998). Establishment and initial characterization of the ovine mammary epithelial cell line NISH. ***In Vitro Cellular and Developmental Biology (animal)* 34**:326-332.

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Abstract

Altering milk composition via transgenesis depends on three main factors. (1) The availability of an efficient regulatory sequences for targeting transgene(s) to the mammary gland; (2) a reliable in vitro model to test the expression of transgenes prior to their introduction to the animal genome; and (3) better understanding of the major factors which determine the rate of gene expression and protein synthesis. The current studies provide the necessary means and knowledge to alter milk protein composition via transgenesis. The following specific goals were achieved:

a: Identifying regulatory regions in the β -lactoglobulin (BLG) gene and the cross-talk between elements which enabled us to construct an efficient vector for the expression of desirable cDNA's in the mammary gland.

b: The establishment of a sheep mammary cell line that serves as a model for the analysis of endogenous and exogenous milk protein synthesis in the mammary gland of livestock.

c: An accurate comparison of the potency of the 5' regulatory sequences from the BLG and whey acidic protein (WAP) promoters in directing the expression of human serum albumin (HSA) to the mammary gland in vitro and in vivo. In this study we have also shown that sequences within the coding region may determine a specific pattern of expression for the transgene, distinct from that of the native milk protein genes.

d: Characterizing the dominant role of ECM in transgene expression in mammary epithelial cells.

e: Further characterization of the BCE-1 enhancer element in the promoter of the β -casein gene as a binding site for the c/EBP- β and Stat5. Identifying its interaction with chromatin and its up regulation by inhibitors of histone deacetylation.

f: Identifying a mechanism of translational control as a mediator for the synergistic effect of insulin and prolactin on protein synthesis in the mammary gland.

Objectives of the original research proposal

1. To define specific cis-acting DNA sequences regions within the BLG gene responsible for reproducibly high level of expression.
2. To define the cellular basis for the apparent different mode of expression of transgene and endogenous milk protein genes.
3. To study cell-cell and cell-ECM interactions that are crucial for the efficient synthesis and secretion of endogenous and exogenous proteins in the mammary gland.

Relevant data, methodology, results and discussion

Milk is a basic nutrient in human and animal food. Modification of milk composition via transgenesis may lead to food of higher quality, save the cheese industry considerable resources, enable distributors to cater niche markets and perhaps aid in generating a healthier livestock. Much is still unknown about the regulation of milk protein gene expression in the mammary gland. Thus, the current project was focused on sequences required for efficient targeting of expression of heterologous proteins to the mammary gland and understanding the basic principals of hormonal and cell- extracellular matrix control of native and foreign gene expression in the gland.

The original specific aims of this research were¹:

1. To define cis-acting DNA sequences regions within the BLG gene responsible for reproducibly high level expression.
2. To define the cellular basis for the apparent different mode of expression of transgenes and endogenous milk protein genes.

¹ The abbreviation used are: BLG - β -lactoglobulin, ECM - Extracellular matrix, EHS - Engelbreth-Holm-Swarm, FCS - Fetal calf serum, HSA - Human serum albumin, MAP K - Mitogen-activated kinase, PHAS-I Phosphorylated heat- and acid-stable protein regulated by insulin, PRL- prolactin, STAT- signal transducer and transactivator of transcription, WAP - Whey acidic protein.

3. To study the cell-cell and cell-ECM interactions that are crucial for the efficient synthesis and secretion of endogenous and exogenous proteins in the mammary gland.

During these studies we also addressed the following additional aims:

1. Analysis of transcriptional mechanisms of extracellular matrix-driven tissue-specific gene expression: Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and ECM and modulated by the state of histone acetylation.
2. Hormonal regulation of native and transgenic milk protein expression.
3. Establishment of a sheep mammary epithelial cell line to serve as a model for the analysis of native and foreign milk proteins in the mammary gland.

Defining cis-acting DNA sequences regions within the BLG gene responsible for reproducibly high level expression.

The promoter of the major milk protein genes can be used to target the expression of any gene to the mammary gland of transgenic animals resulting in the production of desired proteins in the milk. Although mice and sheep carrying genes composed of genomic sequences of human $\alpha 1$ antitrypsin or HSA behind the BLG promoter secrete high level of the corresponding proteins, almost no expression was detected in the mammary gland of transgenic mice carrying the cDNA of these genes.

In the generation of transgenics, the use of vector comprised of a cDNA sequence encoding the desired protein has certain advantages over the use of vector comprised of genomic sequences. The cDNAs are usually more available than the genomic sequences and their significant shorter length makes vector construction easier. However, their poor performance in directing protein expression in transgenic mice, attributed to the increased sensitivity to chromosomal position effect and difficulties in maintaining active chromatin configuration has been problematic. The present study was undertaken to design a BLG vector that enables efficient expression of cDNAs in the mammary gland by identifying regulatory sequences and cross-talk between elements in the BLG gene with the reporter - the

luciferase cDNA. The basis for this study was a previously prepared BLG expression cassette in which cDNA's were inserted between intron1 and 6 of the BLG gene.

Here, the BLG/luciferase constructs were stably transfected to CID-9 mammary epithelial cells that served as model for the differentiating mammary gland. Analysis of luciferase expression was performed in cells cultured on laminin rich matrix (Matrigel) for 6 days in the presence of insulin hydrocortisone and prolactin. (Fig 1). Additional experiments were carried out to determine the relative contribution of each hormone to the luciferase activity.

Regulatory elements in BLG intron 1 and the role of SV 40 PA sequences.

Construct 907 is composed of the original BLG expression cassette (Barash, I., Nathan, M., Kari, R., Ilan, N, Shani, M. and Hurwitz, D.R. *Nuc. Acids. Res.* 24:602-610, 1996) containing the luciferase reporter cDNA inserted between intron 1 and 6. Comparing the expression of construct 907 vs. 914, shows that luciferase activity, when driven by elements of the BLG gene (i.e. BLG promoter, and exon 6 and 3' flanking region), depends on the presence of BLG intron 1. In its absence, there is a dramatic decline in activity.

Surprisingly, a tenfold increase in luciferase activity was detected when the BLG cassette contained sequences of the SV40 polyadenylation (PA) site, inserted downstream of BLG exon 6 (see construct 904). The luciferase activity induced by this construct served as a reference (100%) for the comparison with all other vectors. The BLG PA sequences in construct 904 were not expected to contribute to the expression of cDNA constructs since the SV40 PA sequences were placed in front of them. However their elimination (constructs 921) resulted in a 2.5 fold decline in the luciferase activity. It could be that the BLG PA sequences and 3' serve, at least in this case, as insulator sequences from the suppressing effects of host DNA sequences at the site of integration. Interestingly, the contribution of the BLG PA sequences in these constructs appears to be specific, since no further decrease in luciferase activity could be detected by eliminating the entire BLG 3' region (construct 906).

Also, it should be noted that BLG intron 1 contributes to luciferase activity even in the context of the SV40 PA site (compare construct 904 vs. 915). This contribution appears to be specific to BLG gene sequences since their substitution

Fig.1 Schematic representation of BLG/Luciferase vectors used to transfect CID-9 cells. Luciferase cDNA inserted in the first untranslated BLG exon 1, or in the second exon of the BLG gene, or behind the complete BLG gene were stably transfected into mammary epithelial cells of the CID-9 line. For determination of luciferase activity, cells were cultured on Matrigel in the presence of insulin, prolactin and hydrocortisone for 5 days. Each analysis was performed in triplicates and values were related to those obtained with construct 904. The presented values are mean of more than 3 independent assays.

with insulin intron 1 sequences did not restore luciferase activity (construct 915 vs. 918).

Regulatory elements within the distal part of the BLG promoter and their interaction with BLG intron 1.

Luciferase activity with constructs containing BLG intron 1, SV40 PA and BLG 3' declined when 1kb of the 3kb BLG promoter was deleted from its distal end (construct 920 vs. 904). However, with constructs devoid of BLG intron 1, deleting 1kb of the BLG promoter resulted in a significant increase of over 3 fold in luciferase activity (915 vs. 925), and even in the absence of BLG 3' sequences (construct 908 vs. 919). Taken together, deleting the distal 1kb of the BLG promoter in construct 925 led to a 3.9 fold higher luciferase activity in transfected CID-9 cells than with construct 904, that serves as our reference. This level of activity with construct 925 was 39 fold higher than with our original BLG cassette (construct 907).

Why deleting the distal end of the BLG promoter results in an opposite effect, depending on the presence or absence of BLG intron 1, remains to be determined. Our working hypothesis is that there is a cross-talk between elements within the distal end of the BLG promoter and within intron 1. Experiments are underway to address this hypothesis by testing a series of new gene constructs carrying various deletions in that region.

It should be noted that only marginal activity was observed when the luciferase cDNA was inserted behind the entire BLG gene (construct 916) and essentially no activity was detected with a promoterless luciferase cDNA construct (construct 910).

BLG elements and hormonal effects in transfected CID-9 cells.

We tested the responsiveness of the various BLG expression cassettes to the inductive effect of lactogenic hormones. To this end we used only the more efficient SV40 PA containing constructs that allowed better evaluations of subtle differences. Table 1 shows comparable rates of induction by hydrocortisone and prolactin.

Table 1. Hormonal effect on the expression of BLG/Luciferase constructs in CID-9 cells.

Construct	Composition				Expression (% of IFP)					
	BLG 5'	BLG int.1	BLG 3'	SV40 enh	Matrigel			Plastic		
	(kb)				I	IP	IFP	I	IP	IFP
904	3	+	+	+	30	48 (18)	100 (52)	18	39 (21)	100 (61)
920	2	+	+	+	29	39 (10)	100 (61)	18	60 (42)	100 (40)
906	3	+	-	+	37	55 (18)	100 (45)	30	32 (2)	100 (68)
915	3	-	+	+	28	50 (22)	100 (50)	6	48 (42)	100 (52)
921	3	+	+/-	+				48	64 (18)	100 (36)
924	3*	+	+	+	10	26 (16)	100 (74)	41	57 (16)	100 (43)
919	2	-	-	+	61	78 (17)	100 (22)			
925	2	-	+	+	8	35 (27)	100 (65)	43	58 (15)	100 (42)

Transfected CID-9 cells were cultured for 5 days in the presence of insulin (I), prolactin (P) and hydrocortisone (F) in various combinations. Luciferase activity was determined for each construct at the termination of the culture period, and is presented as percent of that achieved in cells treated with IFP. In parenthesis - the relative contribution of prolactin and hydrocortisone to luciferase activity. +/- lacking only the BLG PA site. * Restricted.

(over the basic activity achieved with insulin alone) with all transfected constructs, independent of the presence of BLG intron 1, or sequences of the distal promoter. This suggests that the differences in expression between the various constructs are not due to altered response to lactogenic hormones. However, while in previous studies we demonstrated major induction of casein and BLG by prolactin with a minor contribution of hydrocortisone, the expression of BLG/luciferase constructs used in this study was highly dependent of the addition of hydrocortisone whether or not cells were cultured on plastic or Matrigel.

Expression in transgenic animals (preliminary analysis):

To test whether elements in the BLG expression cassette that function in vitro can also operate in vivo, transgenic mice carrying constructs 907 and 904 were generated.

None of the 5 transgenic lines carrying construct 907 expressed detectable levels of the luciferase RNA, and marginal luciferase activity was detected in the mammary gland of only one line.

From 3 transgenic lines carrying construct 904 two expressed the luciferase RNA and one had a very high luciferase activity in the mammary gland (about 1000 fold over control level). These results suggest that the SV40 PA sequences contribute also to the in vivo activity of the BLG expression cassette to direct expression of cDNA to the mammary gland. They also suggest that stable

transfected CID-9 cells may serve as a model to test the potential of BLG based constructs to be expressed *in vivo* prior to their introduction into the mammalian genome.

The cell-cell and cell-ECM interactions that are crucial for the efficient synthesis and secretion of endogenous and exogenous proteins in the mammary gland: Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation (Ref. 1).

We (M.J.B) have shown that ECM regulates selectively the set of genes expressed in mammary epithelial cells. To understand the ECM signal transduction pathway leading to tissue-specific gene expression in functionally normal mammary epithelial cells, the β -casein gene, has been studied. This gene is a well known tissue-specific gene encoding for a milk protein and is used as a marker of epithelial differentiation. The first ECM-responsive element to be discovered was isolated in the 5' regulatory sequence of the β -casein gene (Schmidhauser, C., Bissell, M.J., Myers, C.A. and Casperson, G.F. *Proc. Natl. Acad. Sci.* 87:9118-9122, 1990; Schmidhauser, C., Casperson, G.F., Myers, C.A., Sanzo, K.T., Bolten, K. and Bissell, M.J. *Mol. Biol. Cell* 3:699-709, 1992). The shortest DNA sequence of this gene able to integrate the integrin-ECM signaling, was shown to be a 160bp enhancer.

The ECM-responsive element of the β -casein gene is recognized by C/EBP- β and STAT-5.

Studying the transcription factors able to bind to the ECM-responsive element, site specific mutagenesis of the ECM-responsive element has been performed. Mutations were generated across the enhancer by sequential replacement of 10bp stretches with an XbaI site flanked by GCs. In the presence of ECM and prolactin, five mutations resulted in a loss of at least 90% activity as compared to the wild type enhancer. These mutations were localized to two separate regions: a 5' region within the first 100bp which spans across a putative C/EBP- binding site and a 3' region

in the last 60bp which overlaps with a putative STAT-5 binding site. Electromobility shift analysis with oligonucleotides to the functionally important regions have been performed. Nuclear extracts contained an activity complexing with a C/EBP- β site. These complexes could be disrupted with either cold excess of this oligonucleotide or a consensus oligonucleotide to C/EBP- β . Furthermore, addition of an antibody to C/EBP- β but not to C/EBP- α resulted in a supershifted complex. These results established that the 5' region of this 160 bp enhancer is required for its function and is able to bind C/EBP- β . Similar experiments have shown that STAT-5 and an as yet unidentified factor are able to bind to the 3' region of this enhancer and are essential for its function. However, despite the strong transcriptional induction by ECM, no differences in *in vitro* binding activity to these regions were observed in extracts from cells cultured in the presence or absence of ECM.

The ECM-responsive element of the β -casein gene functions only in a chromatin context.

By transient transfection analyses we screened several enhancer-promoter constructs which when integrated into the genome were known to respond either positively or negatively to the presence of ECM. In these experiments, the ECM-responsive enhancer and the MMTV enhancer were linked to a minimal MMTV promoter. ECM did not enhance transcription on a transient template though endogenous β -casein was differentially expressed under these culture conditions as shown by immunoprecipitation of milk proteins. The requirement for stable integration of the ECM-responsive enhancer as well as the known role of chromatin reorganization in MMTV induction, led us to analyze the role of chromatin structure in ECM-induced transcription. We conducted a number of *in vivo* exonuclease footprinting analyses in collaboration with the laboratory of Dr. Gordon Hager (NCI, NIH). We examined chromatin in nuclei isolated from transfected CID-9 cells which were cultured in conditions of active or inactive transcription. To ascertain the transcriptional state of the cells, we routinely performed CAT assays on aliquot of cells taken before the nuclei were isolated. Nuclei were treated with different restriction enzymes to study either the 5' or the 3' region of the enhancer. Stops have been identified within the boundaries of C/EBP- β and STAT-5, however, no differences in the stop patterns were observed under any of the culture conditions tested. This exonuclease study indicates that the three transcription factors are constitutively loaded on their chromatin template, regardless of the ECM presence.

β -casein transcription is turned on by inhibitors of histone deacetylases.

Treatment of the mammary epithelial cell line CID-9, stably transfected with the ECM-responsive element, with sodium butyrate on plastic culture dishes induced transcriptional activity in the absence of ECM. Since sodium butyrate treatment has been shown to induce many changes in the cell in addition to inhibition of histone deacetylase, we tested a new specific inhibitor of histone deacetylase, trichostatin A. Trichostatin A is able to bind directly to histone deacetylase enzymes and works at nanomolar concentrations. A dose-dependent induction of transcription was also observed with trichostatin A in cells cultured in the absence of ECM. As a control of specificity, we have shown that the transcription of a MMTV construct stably-transfected in CID-9 cells was shut down.

Perspectives.

We are now in a position to ask what is the mechanism by which ECM modulates chromatin structure, leading to gene activation and differentiation in our model system?

The results mentioned above indicate that the ECM-responsive element can be activated without ECM, if the ECM signaling is bypassed at the histone level. We have now shown that histone H4 hyperacetylation precedes and correlates with the ECM-responsive element activation, using specific antibodies directed against acetylated lysines (Pujuguet et al., in preparation). We are also investigating the possibility that ECM itself regulates the enzymes regulating the histone acetylation status. Preliminary results indicates that the intranuclear level of HDAC1, the only known enzyme able to deacetylate histone, is downregulated in differentiating cells. Furthermore, transfection of HDAC1 in functionally normal mammary epithelial cells abrogates their ability to differentiate in the presence of ECM (Pujuguet et al., in preparation).

Regulation of transgene expression by cell - extracellular matrix interactions: The BLG/HSA fusion genes do not respond accurately to signals from the extracellular matrix in mammary epithelial cells from transgenic mice (Ref. 2).

Previously, we demonstrated that non-HSA -secreting transgenic strains retain the potential to express the HSA transgene in vitro and that mammary epithelial cell

cultures from non-HSA secreting strains express higher levels of HSA when grown on tissue culture plastic than they do when grown on collagen. In this study, we studied the expression of BLG/HSA fusion genes in epithelial cell cultures of additional transgenic strains and additional substrata. Our results show that: (1) the BLG/HSA fusion gene in only one of seven HSA-secreting or nonsecreting transgenic strains tested, responded to signals from the EHS matrix. (2) HSA DNA sequences dominantly affected the activity of BLG as well as the whey acidic protein (WAP) promoters and (3) HGF/SF induced both milk proteins and HSA gene expression.

These results suggest that the response to the ECM plays a key role in the expression of BLG/HSA fusion genes and that the function of the regulatory elements within the promoter regions of milk protein genes, involved in response to the ECM, in developmental and in tissue specificity, very much depend on the downstream gene sequences.

Hormonal regulation of native and transgene expression (Ref. 3,4).

Activation of milk protein genes in mammary cultures depends on the synergistic effect of lactogenic hormones. This dependency has also been established in the activation of transgenic proteins encoded by hybrid genes composed of native milk protein promoter and the coding sequences from another source. While differences were reported in the requirement of hydrocortisone and prolactin for the activation of WAP and β -casein genes in rat mammary organ cultures, insulin is considered the basic prerequisite for the induction of milk proteins in rodent (except for rabbits) and bovine cultures.

Although the metabolic pathways of insulin and prolactin are under intensive investigation, their synergistic role in stimulating milk protein expression remained an enigma. We have previously generated lines of transgenic mice in which the expression of the HSA gene or minigene is directed to the mammary gland by the sheep BLG promoter. The hybrid BLG/HSA gene switch on their responsiveness to lactogenic hormones during development and a similar pattern of responsiveness to insulin hydrocortisone and prolactin was seen in organ culture. The exceptional feature of BLG/HSA gene expression compared to the endogenous milk proteins is

its relatively high level of expression in the mammary gland of virgin mice. This early developmental expression enabled us to distinguish between transcriptional and post-transcriptional regulation during early pregnancy.

In mammary organ cultures of transgenic mice carrying several BLG/HSA transgenes we have observed, in this project, a dramatic uncoupling of the expression of the HSA RNA and protein. Upon explantation, both HSA RNA and protein were expressed at high levels. However when the explants were grown in hormone-free medium, HSA RNA continued to accumulate, whereas the synthesis of the corresponding protein was dependent on the presence of insulin and prolactin with minor contribution of hydrocortisone. The untranslated HSA RNA was indistinguishable from its translatable counterpart in its mobility on agarose gels, was transported normally from the nucleolus to the cytoplasm and was translated efficiently in rabbit reticulocyte lysate. In the presence of cycloheximide, an inhibitor of protein synthesis, HSA RNA rapidly disappeared, suggesting a dependency on ongoing protein synthesis. Its estimated half-life of 5-6 h in hormone free medium increased significantly in the presence of insulin hydrocortisone and prolactin and was comparable to that of β -casein RNA.

The uncoupling of the expression of the BLG/HSA transgenes at the RNA and protein levels was also confirmed by in situ hybridization and immunohistochemistry of sections from virgin mammary explants. HSA synthesis was initiated within 13 h of the addition of insulin and prolactin in explants that had accumulated untranslated HSA RNA and was fourfold higher than that observed with insulin alone. Addition of hydrocortisone contributed to an additional 20% in HSA synthesis. Interestingly, HSA translation response is much quicker than the onset of β -casein transcription mediated via the binding of MGF (Stat5a) to promoter sequences in HC-11 cells or in COS cells transfected with cDNA of MGF and PRL-receptor. We believe that this was the first demonstration of translational control of exogenous milk protein gene expression in the mammary gland of transgenic animals.

Prolactin and insulin synergize to regulate the translation Modulator PHAS-I via mitogen-activated protein kinase-independent but Wortmannin- and Rapamycin-sensitive pathway.

To probe further into the synergistic effect of insulin and prolactin on protein synthesis in the mammary gland we have initiated a study with differentiating CID-9 mammary epithelial cell line.

It has been shown that the JAK2/Stat5 pathway mediates PRL transcriptional signal transduction leading to the initiation of milk protein synthesis. The binding of STAT5 to specific motifs in the promoters of caseins, BLG and WAP genes promote gene transcription. However, it has been shown in the HC11 mammary cell line that PRL activation of Stat5/MGF is insulin independent, whereas the expression of the β -casein gene depends on the presence of insulin and hydrocortisone.

In addition to the JAK2/Stat5 pathway, PRL stimulation of cellular function has been demonstrated to involve several other Tyr and Ser/Thr phosphorylation pathways including those catalyzed by MAP kinase, S6 kinase, Raf-1, protein kinase C phosphoinositol (PI) 3'-kinase and fyn. However no conclusive evidence for synergism with insulin could be drawn from these studies dissecting these pathways.

Recently, it has been shown that insulin initiates protein translation activity in NIH 3T3 cells and fat tissue by inducing the phosphorylation of PHAS-I, a binding protein to the translation initiation factor eIF-4E. The phosphorylation of PHAS-I, results in its dissociation from the eIF-4E, enables binding of eIF-4G and allow the formation of an active complex which induce the initiation of translation by augmenting mRNA cap binding ability to binding the small ribosomal subunit.

Our working hypothesis was that the synergism between insulin and prolactin on protein production in the mammary gland may be mediated via the regulation of protein translation. This hypothesis was studied in differentiating mammary epithelial CID-9 cells cultured on laminin-rich matrix and treated with insulin alone for 5 days. Prolactin was added to the insulin treated cells on the 6th day for 0 -180 min.

We demonstrated that the phosphorylation of PHAS-I depends on the presence of both Insulin and prolactin in the culture medium and resulted in the dissociation of PHAS-I from the eIF-4E translation initiation factor. This step is crucial for the initiation of translation. The induction of PHAS-I phosphorylation was rapid and its rate matched that demonstrated for the JAK2/STAT5a and the binding of STAT5a to its DNA binding motif. However, 120 min were needed for complete phosphorylation of PHAS-I, evidenced by the shift of the protein to its highly phosphorylated, slower migrating γ form.

In the presence of insulin, PRL induced MAP kinase activity at a comparable time course to that of PHAS-I phosphorylation. However, a line of evidence suggested that although this kinase phosphorylates PHAS-I *in vitro*, it does not actively participate in its phosphorylation *in vivo*: (a) the level of insulin needed to enable PRL-induced ERK-1/ERK-2 activation was one order of magnitude higher than that needed for PHAS-I phosphorylation and (b) PD 098059, a MEK-1 inhibitor, completely inhibited insulin-dependent, PRL-induced ERK-1/ERK-2 activation but had no effect on PRL-induced PHAS-I phosphorylation. In contrast, wortmannin, a PI 3' kinase inhibitor, and the immunosuppressant rapamycin abrogated PHAS-I phosphorylation and caused a reciprocal shift between the fully phosphorylated PHAS-I γ form and its non-phosphorylated α form. We speculate that the synergism between insulin and PRL in activation of PHAS-I phosphorylation is mediated via their effects on the IRS1 and/or PI 3' kinase. Since the partly phosphorylated PHAS-I β form was not significantly affected by these inhibitors, it is possible that this synergistic effect is generated by two kinases.

In vivo and in vitro expression of HSA genomic sequences in mammary epithelial cells with BLG and WAP promoters The apparent different mode of expression of transgenes and endogenous milk proteins (Ref. 5).

The control elements of the major milk protein genes are being exploited to target the expression of foreign genes to the mammary gland of transgenic animals. In search for *in vitro* models for the efficiency of transgene expression in the mammary gland, we have compared the *in vivo* and *in vitro* expression of HSA genomic sequences in mammary epithelial cells with the BLG and WAP promoters. We also characterized the apparent heterogeneous pattern of the HSA transgene expression observed in BLG/HSA transgenics and compared it with the *in vivo* pattern of expression of same HSA genomic sequences driven by the rat WAP promoter. Previously, we have demonstrated heterogeneous pattern of expression of the BLG/HSA genomic sequences in the mammary gland of transgenic mice as opposed to the homogenous pattern of expression of the native milk protein genes.

The expression pattern of HSA in transgenic mice carrying various HSA genomic sequences driven either by the mouse WAP or the sheep BLG promoters, was compared. The pattern of HSA expression in both WAP/HSA and BLG/HSA transgenic lines was copy number independent, and the major site of ectopic expression was the skeletal muscle. Although an equal proportion of expressors was determined in both sets of mice (~25% secreting >0.1 mg/ml), the highest level of HSA secreted into the milk in the WAP/HSA transgenic lines was one order of magnitude lower than in the BLG/HSA lines. Despite this difference, the HSA expression patterns in the mammary gland was similar and consisted of two levels of variegated expression, correlated with the number of copies of the transgene. Studies using mammary explant cultures revealed a comparable responsiveness to the lactogenic hormones insulin, hydrocortisone and prolactin, although the WAP/HSA gene constructs were more sensitive to the hydrocortisone effect than were the BLG/HSA vectors. When HSA vectors were stably transfected into the mouse mammary cell line CID-9, they displayed a hierarchy of expression, dependent upon the specific complement of HSA introns included. Nevertheless, the expression of HSA in four out of five WAP/HSA constructs was similar to their BLG/HSA counterparts. This construct-dependent, and promoter-independent, hierarchy was also found following transfection into the newly established GOLDA-1 ovine mammary epithelial cell line. With the increasing importance of culture models to test gene constructs prior to the formation of transgenic animals, the in vitro/in vivo comparisons of the WAP/HSA and the BLG/HSA gene expression were informative. While the proportion of expressors were the same, the 10-fold difference in expression in vivo was eliminated in culture. Furthermore, transfection of CID-9 and GOLDA-1 cells with the various HSA vectors may provide further information about putative regulatory elements within the HSA gene sequences.

Establishment of a sheep mammary epithelial cell line to serve as a model for the analysis of endogenous and exogenous milk protein synthesis in the mammary gland. Establishment and initial characterization of the ovine mammary epithelial cell lines NISH and GOLDA-1 (Ref. 6).

Many tissue culture models (organ cultures, primary cultures, mammary epithelial cells and cell lines) have been established to better understand the complex biology of the mammary gland. For years, the only functional and well-characterized cell line available was the COMMA-1D cell line, derived from normal pregnant mice. The COMMA-1D and its subclones HC-11 and CID-9 cell lines were used extensively to study the effect of lactogenic hormones and ECM on milk protein gene expression. Although, many other mammary epithelial cell lines have been established from human and rodents and contributed to our knowledge of mammary gland physiology, very few cell lines are available from the major milk protein producers, the farm animals. The establishment and study of mammary epithelial cell lines from farm animals is of special interest because the rodent model does not accurately reflect udder development and physiology. No mammary cell lines have been established from sheep or goat, and only four bovine cell lines are currently available. None of these cell lines show both hormonal and ECM response and only one of them (BME UV) shows marginal casein synthesis. These cell lines are therefore unsuitable for the study of the effect either of the hormonal regulation of ovine/bovine casein expression or the effect of ECM on milk protein gene expression.

In the current study we established an heterogeneous functional epithelial cell line (NISH) and its sub-clone GOLDA-1 which may represent the stem cells of the mammary gland. When plated in or on top of the appropriate ECM, both NISH and GOLDA-1 cells secrete BLG (Golda -1 was analyzed also for β -casein synthesis and found positive) in an ECM dependent manner, and are capable of forming structures resembling ducts, lateral buds and alveoli. Studies were completed for NISH cells and are currently continued for the GOLDA-1 clone.

Interestingly, the presence of growth hormone dramatically increased BLG secretion from NISH cells cultured on ECM. It appears that GH is required not only to establish the structural organization, but is also continuously needed to maintain BLG expression in these cells. Stable transfection of NISH cells with BLG /HSA gene constructs revealed that the relative level of expression was comparable to the *in vivo* secretion of HSA in transgenic mice carrying these gene sequences. No transcripts could be detected in cells transfected with hybrid genes carrying either

HSA cDNA or the entire HSA gene, and HSA expression was dependent on the presence of intronic sequences.

Some minor differences were observed between the heterogenous population of NISH cells and its sub-clone GOLDA-1 which demonstrated the same morphology as shown for the NISH when cultured on matrix, but was less dependent on GH for organization and milk protein synthesis. Golda 1 cells were also successfully transfected with both BLG/HSA and WAP/HSA gene constructs and displayed an hierarchy of expression, dependent upon specific complement of HSA introns included.

These results demonstrate that NISH and GOLDA-1 cells may provide useful tools for studying the differentiation and organization of mammary epithelial cells under defined culture conditions. Furthermore, transfection of these cells may be an alternative for the transgenic mouse model in evaluating the potential of gene constructs to be efficiently expressed in the mammary gland of transgenic farm animals.

Evaluation of the research achievements as related to the original research proposal

All the aims of the original research proposal were addressed. We have made progress in identifying regulatory sequences in the BLG gene and pointed towards a possibility of cross-talk between those elements. The dominant role of the ECM in regulating transgene expression was investigated and the special in situ pattern of transgene expression in the tissue was demonstrated.

In addition, we have made progress in the study of three related subjects that were not part of the original proposal: (1) Analysis of transcriptional mechanisms and characterization of a transcriptional enhancer in the promoter of the β -casein gene. (2) Hormonal regulation of endogenous and transgene expression in the mammary gland with a focus on a translational regulation. (3) Establishment of a cell line from the mammary gland of a farm animal to serve as a model for studying milk protein gene expression in the gland of ruminants. Overall, the results of these studies were published in 6 manuscripts.

Description of cooperation

The US group cloned and characterized the CID-9 mammary epithelial cells and made them available to the Israeli group. Here, these cells served as a model to analyze the feasibility of transgene expression prior to its introduction to the host genome and the production of transgenic animals. The US group identified the BCE-1 element in the promoter of the β -casein gene and shared the knowledge and techniques with the Israeli group who focused on the BLG gene. Together, we have published one manuscript (#5 in the references list) and share the unpublished results concerning identifying elements in the BLG gene.

Conclusions

Altering milk composition via transgenesis is now feasible for any cDNA using the BLG expression cassette. The pattern and the level of transgene expression is determined by the regulatory sequences derived from the milk protein gene, but is also affected by sequences within the region, the structure of the chromatin at the site of integration and the interactions between the epithelial cells and the ECM. Indeed, the ECM which is essential for native gene expression in the gland, may also exert negative effects on transgene expression. In vitro culture systems, derived from mammary cell lines of mouse or ovine mammary gland, are valuable in evaluating regulatory sequences, both in the promoter and in the coding region. However, these models do not accurately mirror the overall pattern and feasibility of transgene expression in the gland.

Publications which resulted from the studies supported by this grant

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