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α-Lactalbumin - The milk manipulator's dream

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ABSTRACT

The properties of the milk serum protein α-lactalbumin are reviewed with special emphasis being placed on its central role in determining the lactose and water content of milk. Much circumstantial evidence suggests that artificial depression of α-lactalbumin synthesis should decrease lactose synthesis and that this will, in turn, decrease the water content of milk. Experimental evidence indicates, however, that patterns of α-lactalbumin gene expression in lactating ovine and bovine mammary tissue are complex and we should be cautious in making such predictions. Studies with transgenic mice expressing either elevated or depressed levels of α-lactalbumin will be required to settle this question. In the meantime, a promising line of research is the investigation of dairy cattle carrying variant α-lactalbumin genes. It is possible that correlations will be found between some of these variants and both the water content and protein yield of milk. Conceivably, selective breeding of cattle carrying variant proteins may prove a more practical means of altering α-lactalbumin synthesis than current transgenic techniques.

Keywords α-Lactalbumin, lactose, water-content, gene-structure, transgenics, gene-expression, variant-proteins.

INTRODUCTION

α-Lactalbumin is a minor component of milk, constituting no more than 4% of total protein and, as such does not have the bulk which makes the major proteins such as the caseins so important in manufacturing industries. Nor does it have the semi-pharmaceutical properties which gives a protein such as lactoferrin enhanced value. Despite this lack of direct economic value, α-lactalbumin does play a supremely important biochemical and physiological role in milk production. It is this role which will be discussed here.

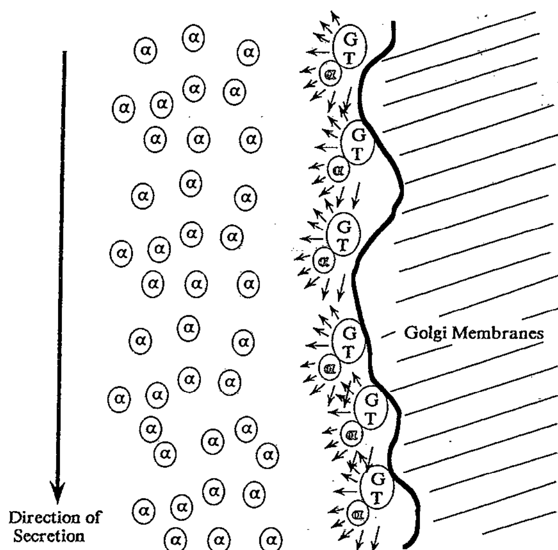
Until 25 years ago α-lactalbumin was just thought of as another milk protein but then Brodbeck *et al.* (1967) made the fundamental discovery that α-lactalbumin can form a transient complex with galactosyltransferase and radically increases the affinity of this enzyme for glucose. Galactosyltransferase is bound to the inner surface of the Golgi apparatus in mammary epithelial cells and catalyses the production of lactose from UDP-galactose and glucose through a series of coordinated and compartmentalised reactions, most of the details of which need not concern us here.

The one point to be emphasised is that, in the absence of α-lactalbumin, the K_m of the enzyme complex for glucose is so high (in the molar range) that virtually no lactose can be synthesised, but once α-lactalbumin is bound into the complex the K_m drops to the millmolar range, which is also the physiological range for glucose concentrations in the mammary cell, and lactose synthesis can take place. Thus, while α-lactalbumin is not an enzyme it is an *effector* molecule which greatly increases the activity of galactosyltransferase under physiological conditions.

LACTOSE AND THE WATER CONTENT OF MILK

Two major points must be borne in mind when considering the water content of milk. First, in virtually all mammals the osmolarity of the milk closely parallels that of the blood, the so-called *isosmolarity* principle and second, lactose is the major osmole in the milk (Mepham, 1987). Logically, one would therefore expect that, in order to maintain osmolarity, a milk of low lactose concentration would have to have a high concentration of other components while for a high lactose concentration the opposite would be true. This does seem to be borne out in practice for a wide range of species, with both protein and fat concentrations showing

an inverse correlation with lactose concentration (Mepham, 1987). There is also some evidence for a correlation between α-lactalbumin and lactose concentrations in milk from a number of species (Ley and Jenness, 1970). Examination of the fluctuations in lactose, protein and fat concentrations within a given lactation in a number of species also suggests that the same inverse correlation exists although there are some unexplained data for human milk which has been artificially expressed (A.L. Parry, *pers. comm.*).



Flow of α - lactalbumin through Golgi.

α = α - lactalbumin molecule

α = α - lactalbumin molecule in effector role on -

GT = galactosyltransferase molecule

⟶ = lactose

FIG 1 Flow of a-lactalbumin through the Golgi. a represents α-lactalbumin molecules; a represents α-lactalbumin molecules in an effector role; GT represents the galactosyltransferase molecule and ——> the release of lactose.

Whether or not these fluctuations in lactose levels results from fluctuations in α-lactalbumin protein levels is not clear, nor for that matter is it clear whether or not all α-lactalbumin molecules are efficient effector molecules or whether those which are glycosylated could be targetted to specific regions of the Golgi apparatus (Paulson, 1989). Whichever is the case, in most mammals α-lactalbumin is produced in considerable excess over that required to saturate the galactosyltransferase complex and one can imagine a percolator type situation (Fig. 1) in which the bulk of the α-lactalbumin molecules flow past the Golgi with, at a given time, only a small fraction forming complexes. Ball-park calculations would suggest that α-lactalbumin levels would have to drop 3 or 4-fold before the galactosyltransferase complex ceased to be saturated with α-lactalbumin, thus one might expect that, if this was the only control on lactose synthesis, quite dramatic fluctuations in α-lactalbumin levels would have to occur before lactose concentrations were greatly altered.

What happens if we were to biochemically intervene in lactation and dramatically depress α-lactalbumin protein levels? One would assume that once the levels were down to 10 to 20% of normal, lactose synthesis must decrease and the osmolarity of the milk would tend to decrease. One would also assume that homeostatic mechanisms would then come into play to maintain osmolarity by either increasing the secretion of ions or by decreasing the secretion of water. In a fully lactating animal such as a cow, the latter scenario is most likely so we would predict that a lower volume of milk, containing elevated protein and fat, would be secreted. Whether or not production of total solids would be maintained under these conditions is obviously a matter of considerable conjecture and any such losses would obviously have to be weighed against the advantages of lowering milk volumes and a more concentrated milk.

WHY MANIPULATE MILK VOLUMES?

Basically, three arguments can be put forward in favour of lowering milk volumes. The first argument involves the storage, transport and processing costs which the water in milk incur. Currently there is considerable interest in developing an on-farm method for extracting water from milk using a mechanical filtration process which concentrates the milk two or three-fold. The Waikato Valley Cooperative Dairies have already invested some $3 million in the project and it is hoped that the first units (the Milcon 100) will be on sale shortly for approximately $35,000 each. They are initially aiming the product at farmers who are in danger

of losing access to dairy factories because of the high costs of servicing their remote locations. It remains to be seen just how technically successful and how cost-effective such an approach will be. The main point to be taken from this story is that reduction of water in milk is a goal to be taken seriously and that, whilst mechanical means such as those just described might be attractive to a few farmers, manipulating milk composition by biochemical means would provide a method of introducing changes across the board which, although perhaps relatively modest, would add up to very significant advantages at the national level.

The second advantage of reducing water content in milk is related to the concept of once-a-day milking (see L'Huillier *et al.*, 1989; Carruthers, *et al.*, 1991). Such a practice is accompanied by a drop in production and, although it would be simplistic to contend that reducing the water content, and the volume, of milk would completely reverse this reduction in yield, it is clear that such a manipulation would tend to overcome any limitations imposed by the storage capacity of the udder.

The third advantage, although not strictly related to milk volume, is that a low lactose milk may have a market in those areas of the world where large fractions of the population are lactose-intolerant. However, it is probably not practical to depress lactose more than 50% or so which might not produce a suitable milk.

How Can α-Lactalbumin Levels be Manipulated?

Essentially this question resolves down to a molecular biology problem because there is really no other practical way of selectively inhibiting a single protein in mammary tissue. Within this context there are three broad possiblities. The first is to use molecular biological techniques to screen animals for "defective" α-lactalbumin gene mutants; there are obvious advantages in such an approach both from the practical, technological and ethical points of view. If, however, suitable α-lactalbumin mutants cannot be found, the alternative is to genetically engineer an animal (first a mouse as an experimental model and then a farm animal) which has either an altered or a deleted α-lactalbumin gene or, taking a different approach, engineer an antisense or ribozyme RNA into the animal which is capable of destroying mRNA synthesised by the the α-lactalbumin gene (L'Huillier *et al.*, 1989).

All three aforementioned approaches require that we know something about the α-lactalbumin gene.

THE STRUCTURE AND FUNCTION OF THE α-LACTALBUMIN GENE

The bovine α-lactalbumin protein consists of 123 amino acids plus an additional leader sequence which is cleaved off during cellular processing. Complete and partial sequences of α-lactalbumin proteins from a number of species have been reported with most demonstrating large amounts of homology, especially in the region which binds to galactosyltransferase. Interestingly, α-lactalbumin shows considerable cross-species functionality as an effector molecule with, for example, that from possums showing some 20% of the efficiency of that from bovine in stimulating lactose synthesis by bovine galactosyltransferase (Grigor *et al.*, 1991).

At the genome level, rat α-lactalbumin was the first to be cloned closely followed by the genes from bovine, ovine, guinea-pig and human. Again, the homologies seen at the protein level are also obvious both at the DNA sequence and organisational level (Gaye *et al.*, 1987), as is a region of the gene with similarities to lysozyme, indicating some evolutionary link. When Southern blots of DNA from other species, such as whale and mouse, are probed with the bovine cDNA discrete bands are seen suggesting that significant homologies also exist with these species (R.J.Wilkins, unpublished data). So far, however, the search for the mouse α-lactalbumin gene has been puzzlingly and frustratingly negative. Results from our own laboratory (P.H.Bissinger, *pers. comm.*) suggest that the search may be confounded by several related sequences in the mouse genome. Certainly in bovine and ovine, there are pseudo-gene sequences related to the α-lactalbumin gene (Soulier *et al.*, 1989). Whatever the reason, a lack of a cloned mouse α-lactalbumin gene has seriously hampered lactational studies in mice whether they be molecular biological, transgenic, embryonal stem cell or cell cultural in nature. This has meant that gene manipulation work has focussed on bovine constructs, even when milk synthesis is being studied in mice and mouse cell-lines.

Perhaps the most important finding to date is that the 700bp of DNA upstream from the bovine α-

lactalbumin is sufficient to ensure high levels of gene expression when constructs are introduced into transgenic mice (Vilotte *et al.*, 1989). In this respect, the control of α-lactalbumin gene expression appears to be somewhat more straightforward than other milk protein genes such as the caseins. This finding considerable simplifies the study of α-lactalbumin gene expression because it means that we can focus most of our attention on these 700bp of DNA.

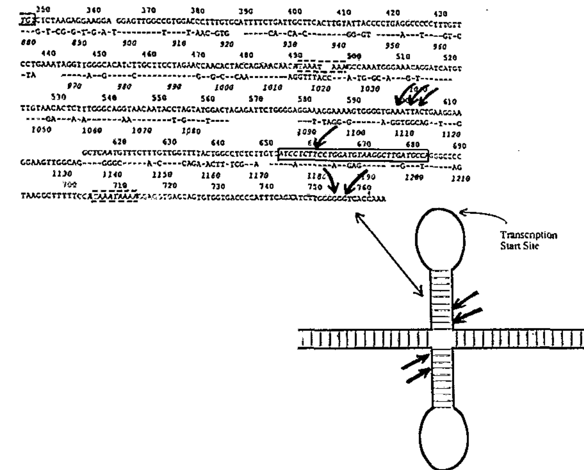


FIG 2 DNA sequence variants found in the upstream region of the bovine α-lactalbumin gene. Arrows indicate regions of base substitutions. The main changes to note are in the progesterone/ lysozyme binding site (boxed) and in the palindromic region defining a cloverleaf (right) around the transcription site. DNA sequence is adapted from Vilotte *et al.* (1987).

In our own work, we have designed an assay which enables us to look for sequence variations in this upstream DNA region. To do this we use two primers to amplify up this region of DNA by using the polymerase chain reaction (PCR) and then we use a series of nested primers and standard sequencing reactions to search for "mutant" base substitutions. So far, our preliminary experiments indicate that a suprisingly large number of variants do exist; in twenty animals tested we found that six had at least one base change (see Fig. 2) and some of these were in important regions of the upstream sequence. Intriguingly, the animals we tested were Angus not dairy cattle! While just what effect these and other "mutations" will have on α-lactalbumin gene expression is not yet clear, the important point to make at the moment is that these "mutations" do occur and rather frequently so it means that out there in the cattle populations, we are likely with patience, to find animals with altered α-lactalbumin expression.

Screening animals for specific mutations can be done rather rapidly using a combination of PCR and allele specific oligonucleotides. Already, such screening methods are planned for looking at genotypic variants of k-casein and β-lactoglobulin so it should be straightforward to extend the studies to α-lactalbumin. So far we have not investigated the possibility of significant structural variants within the α-lactalbumin protein itself and, although some variants have been reported, non have been correlated with production traits. One approach we are considering is the artificial production and functional testing of α-lactalbumin variant proteins using *in vitro* mutagenesis so as to determine the screening assays we should use to cover the regions of the α-lactalbumin gene in which mutations are going to have the most profound effects.

Transgenic Experiments with the α-lactalbumin Gene.

Two kinds of experiments are possible with transgenic mice. The first and the most direct would be to ablate the endogenous α-lactalbumin gene by using embryonal stem cell techniques (Wagner, 1990). Although this is probably the most extreme experiment possible it might well give no information other than a demonstration of how lactation is aborted when an animal attempts to produced lactose free milk and, in any case, it cannot be done until the mouse α-lactalbumin gene is isolated. The second approach is to introduce foreign gene constructs into transgenic mice, the most obvious of which is bovine α-lactalbumin. At least two groups have done this (Vilotte *et al.*, 1989; R.D.Bremel, *pers. comm.*) and achieved bovine α-lactalbumin protein levels almost comparable to endogenous expression; in our case we have produced a number of transgenics which have integrated the bovine cDNA together with a CMV promoter and enhancer but, as yet, none of these have been found to express the bovine protein (L'Huillier P.J., Davis S.R., Wilkins R.J. and Thompson J.G.E., *pers. comm.*). Detailed studies have not been reported on the composition of milk in these transgenics, but

Bremel does have some very preliminary data which suggests that a greater volume of more dilute milk may be produced from some of his transgenic mice.

An alternative approach, using ribozymes and antisense RNA to modulate α-lactalbumin mRNA is reported by L'Huiller, Bellamy and Davis (1991) at this meeting. Although their experiments are currently restricted to a model *in vitro* cell culture system, they are most promising and indicate that a very high fraction of bovine mRNA can be cleaved. So far there are no reports of such a system working in mammary tissue although a ribozyme for acetyl CoA carboxylase which has been coupled to the α-lactalbumin upstream region does show considerable promise (Bremel R.D., *pers. comm.*).

Patterns of Expression of the α-Lactalbumin Gene.

In all the discussion so far, the tacit assumption has been made that when a milk protein gene is switched on in lactation it will be expressed uniformly in all mammary tissue - or more strictly, all alveoli. To test this expectation we have initiated a series of *in situ* hybridisation experiments in order to detect which cells in mammary tissue sections are actually expressing α-lactalbumin mRNA. These experiments which are reported elsewhere at this meeting (Molenaar *et al.*, 1991) show that the patterns of expression are, in fact, complex and far from homogeneous. In sheep in late pregnancy, expression is turned on in some regions of the gland while other, histologically similar regions, have little or no expression. During mid-lactation there are again distinct differences with collapsed or "milked out" alveoli giving high expression while those full of fat globules (both in the lumen and epithelial cells) show little expression. Our limited data on dairy cattle suggest a somewhat similar pattern.

These data raise a whole host of questions - does rapid cycling of gene expression occur and is it in some way related to milking regimes, are we looking at early events in involution, do the patterns of expression vary significantly from animal to animal, breed to breed and species to species, does the heterogeneous expression of the α-lactalbumin gene expression reflect a mechanism which drives morphological changes or is it simply a result thereof? Do other milk protein genes show heterogeneous patterns of expression (we know that in mice, at least, the casein gene expression is homogeneous - M.J.Bissell, *pers. comm.*)? Some of these questions are relatively easy to address, others much more difficult. These preliminary data do, however, enable us to make two basic observations. First, there must be a subtle means of local control over endogenous α-lactalbumin gene expression within the mammary gland and, second, this presumably influences local levels of lactose synthesis.

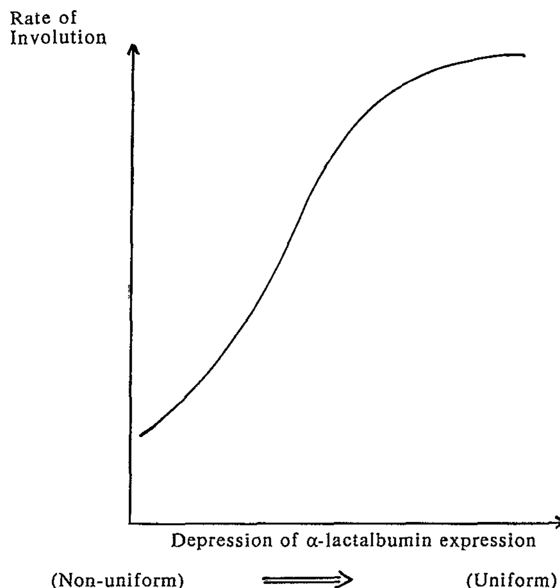


FIG 3 Schema of how involution may be influenced by a transgene which uniformly either increases or decreases α-lactalbumin gene expression.

Problems with α-Lactalbumin Transgenics?

Given these data, what can we expect a transgene for α-lactalbumin to do? If we design the gene so that it is always strongly expressed we will obviously destroy the heterogeneous patterns of gene expression in the mammary gland, and even if we do attempt to mimic the *in vivo* control this may still be the case because the controlling elements in the transgene are very unlikely to be subject to the same signals which modulate the local expression of the endogenous gene. Thus, in the extreme case, all regions of the mammary gland will now be actively synthesising lactose with a comcomitant

increase in water content and milk volume. In otherwords, a relatively modest but uniform increase in transgene α-lactalbumin synthesis throughout mammary tissue, which would normally not be expected to have much of an additional effect on the already activated galactosyltransferase complexes in cells could actually have a quite profound effect if the some of these cells are normally starved of endogeneous α-lactalbumin. Perhaps this is the basis of Bremel's unexpected observations of increased milk volume in transgenic mice.

If a causative mechanism is actually involved one also wonders if a uniformly high transgene expression of α-lactalbumin would prevent involution - and would a complete shutdown of α-lactalbumin speed it up (Fig. 3) ? These are all questions that transgenics alone will answer.

FUTURE RESEARCH DIRECTIONS

It is clear that research into the manipulation of α-lactalbumin is still in its infancy. We can really proceed in two directions, either a "non-interventionist" approach in which we use DNA techniques to screen and identify animals which have unusual α-lactalbumin genes and / or controlling elements, or we attempt to engineer animals which have artificially altered α-lactalbumin, lactose and water levels in their milk. The former approach can be implemented right now and, although it is unlikely to yield dramatic results it could well result in the breeding of animals with a few percent difference in the aforementioned "traits." That is, it could well have an economic potential similar to screening procedures aimed at selecting favourable genotypes of k-casein and β-lactoglobulin.

In the meantime it is clear that serious efforts to produce farm animals transgenic for α-lactalbumin would be premature until we know muchmore concerning the effect of such a transgene on mammary function in a mouse model. How is water content affected, is involution speeded up if α-lactalbumin expression is depressed in all cells by a ribozyme attached to a strong promoter, does the uniform overexpression of α-lactalbumin increase water secretion and slow down involution, is it necessary to develop a transgene promoter which mimics exactly the alveoli specific expression of the endogenous α-lactalbumin gene? These questions could take several years to answer.

Once answers are found from mouse transgenics, the application of the results to dairy goats and cattle on any kind of moderate scale could take ten years or more even if there are no technical problems, simply because of the time required to breed herds that stably express the gene. Added to this is the relative inflexibility of the procedures in that one is committed to a particular gene construct and transgenic insertion for many many years. All is not gloom and doom, however, in that our methods of making transgenics are likely to improve dramatically over the next ten years and techniques of injecting DNA *in vivo* into animal tissue and having it stably expressed for several months (Wolff *et al.*, 1990) could be ideally suited for use with mammary tissue (Patton *et al.*, 1984) especially if transformation was possible at the stem cell stage. Looking even further into the crystal ball, it might be possible to programme a cow for modified gene expression from lactation to lactation simply by injection of the appropriate gene construct. This would have several advantages e.g. complete flexibility and responsiveness to market demands from season to season, reversiblity of the transgene "genotype" at anytime and avoidance of the practical and ethical problems associated with altering the gene pool.

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