Localisation of α-lactalbumin gene expression in sheep mammary tissue

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ABSTRACT

Mammary tissues from virgin, prepartum and lactating ewes were examined by *in situ* hybridisation with a ^{35}S labeled cRNA probe derived from the bovine α-lactalbumin gene. Expression of α-lactalbumin was absent in the virgin udders. In the pregnant gland, α-lactalbumin expression was found in some secretory alveoli but not in others, even though they appeared histologically similar, containing milk or colostrum and fat globules. Fully lactating ewes had several levels of α-lactalbumin expression. In secretory epithelium, it was high in collapsed thick walled lactating alveoli, slightly less in distended thin walled lactating alveoli with homogeneous contents and absent from alveoli containing an abundance of large fat globules. β-cytoplasmic actin expression was the reverse of this. These observations are intriguing and suggest a number of hypotheses. Firstly; that α-lactalbumin gene expression is linked to the long term secretory activity of cells and drops off once cells are resting or regressing. Secondly; that α-lactalbumin gene expression is highest in those alveoli which are "filling up" and lowest in those which are full or have just been milked out. That is, there are cyclical variations in expression. And thirdly; that there is distinct compartmentalisation in the lactating gland, and synthesis of α-lactalbumin mRNA (and the protein?) occurs in cells which synthesize little fat.

Keywords α-lactalbumin, milk gene expression, *in situ* hybridisation, mammary.

INTRODUCTION

The mammary gland is a complex organ both in structure and function. It undergoes three physiological transitions during a lactation cycle; from involution to colostrogenesis, from colostrogenesis to lactation, and from lactation to involution. Marked changes in mammary size, structure and secretions occur as the gland progresses to or from a state of active milk synthesis. (Oliver and Sordillo, 1989; Saacke and Heald, 1974).

α-Lactalbumin, one of the two subunits of lactose synthetase, catalyses the conversion of glucose and galactose into lactose (Gordon, 1971; Qasba and Safaya, 1984; Mepham, 1987). The synthesis of lactose is thought to be involved in the regulation of water movement into milk (Linzell and Peaker, 1971; Nickerson and Akers, 1984) as lactose is the major osmotic component of milk.

A number of studies have been performed quantifying and temporalizing the overall expression of various milk protein genes in the mammary gland (Burditt *et al.*, 1981; Nardacci *et al.*, 1978; Nakhasi and Qasba, 1979). Interest has also been shown in whether all cells produce all proteins (Burditt *et al.*,) at all times. The current dogma is that all active cells produce all the components of milk as the generation of cell membrane used to encapsulate protein before exocytosis, is needed to replace cell membrane lost when fat globules are released into the lumen (Nickerson and Akers, 1984; Cowie and Tindal, 1971; Linzell and Peaker, 1971). More recent calculations suggest that rates of membrane removal/replacement are not equal (Mepham, 1987).

Using *in situ* hybridisation we report that α-lactalbumin expression in sheep changed dramatically with lactational status, was not uniform throughout the tissue, and varied in differing types of secretory alveoli.

MATERIALS AND METHODS

Tissues

Mammary tissues from four virgin, two 14 days prepartum and four one week post partum lactating ewes, were removed from the animals immediately after exsanguination, fixed for one day in phosphate

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buffered formalin then wax-embedded using standard histological methods.

In situ Hybridisation.

Five µm sections were cut, floated onto 0.2% gelatin in water and mounted onto clean slides, dried at 37 to 50°C, dewaxed and then permeabilised by incubation in 0.2M HCl for 10 min, neutralized in 2X SSC (0.3M NaCl, 0.03M Na Citrate) for 30 min followed by a 15 min incubation with 2 ug ml^{-1} Proteinase K in 0.2M Tris-HCl pH 7.2, 2mM $CaCl_2$ at 37°C. For the hybridisation, 10ul cm^{-2} of hybridisation mix (50% formamide, 2X SSC, 0.15M NaCl, 0.2 mg ml^{-1} *E.coli* tRNA, 1 mg ml^{-1} degraded herring sperm DNA, 0.1 mg ml^{-1} BSA, 10% Dextran Sulphate, 0.2M DTT) was spread over the dried section and incubated at 50°C for 30 min. This was then replaced with 5ul cm^{-2} of the hybridisation mix containing 1-50 x 10^3 cpm/µl of sense or antisense transcript and incubation continued overnight at 50°C. Slides were then washed in 2 changes of 50% formamide/2X SSC at 52°C for 5 and 20 min, rinsed 3X in 0.2X SSC at RT, once in 2X SSC at room temperature, incubated in 100 ug ml^{-1} RNAse A, 1µg ml^{-1} RNAse T1 in 2X SSC for 15 to 30 min at 37°C, washed again in 0.2X SSC several times with gentle agitation then dehydrated in ethanol and air dried. All washes contained fresh 10mM β-mercaptoethanol. Autoradiography was with Kodak NTB^{-2} or diluted 1/1 Ilford K5 emulsion for 1 to 6 weeks. Slides were stained with rapid haematoxylin and eosin.

RNA Probes.

The 700 Base pair α-lactalbumin cDNA (Hurley and Schuler, 1987) was excised and cloned into the Invitrogen expression vector pRc/CMV in both orientations. Sense and antisense RNA transcripts were synthesised from linearised templates using the Promega riboprobe kit with 0.4 ug DNA and were labeled with 2.5 µl of >1000 Ci mmol L^{-1} ^{35}S UTP (Amersham) (25 µCuries). Transcripts were DNAsed, precipitated, and stored frozen in 20mM DTT at a radioactive concentration of approximately 10^6 cpm ul^{-1}. Sense transcripts gave very low and non specific binding over all tissues. β-cytoplasmic actin is a cytoskeletal element present in all cells and its mRNA is found in all viable cells. It is often used as a control to prove that RNA is present, relatively intact and detectable in the cell. Here, the human cytoplasmic β-actin probe (700bp EcoRV-Ball fragment of the 2 kb human actin cDNA (Gunning *et al.*, 1983)) was used to check for the presence of mRNA in the sections.

RESULTS AND DISCUSSION

FIG 1 'Virgin' mammary.
A; α-Lactalbumin signal, 400x. B; β-Actin signal, 400x.
Expression is seen as small black dots.

In virgin glands (Fig. 1A), no α-lactalbumin expression was detected. The detection of β-actin mRNA in the 'virgin' mammary sections (Fig. 1B) verifies that α-lactalbumin mRNA would have been detected if present.

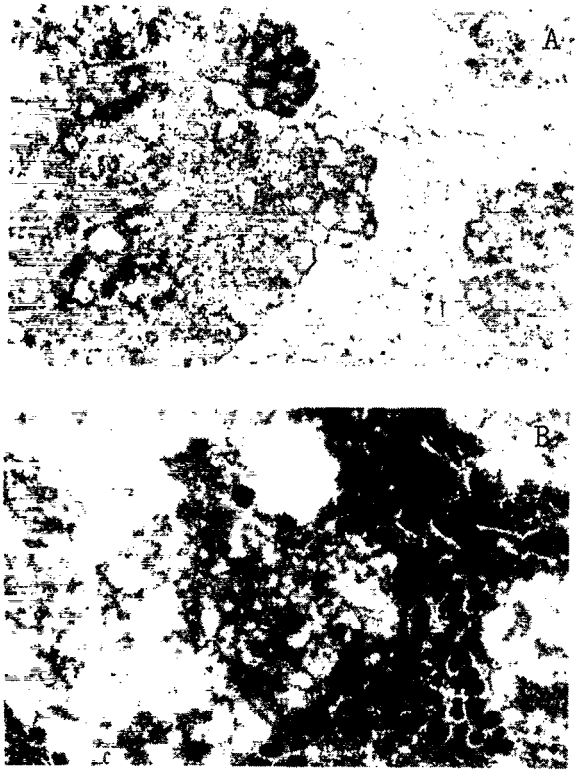

FIG 2 'Pregnant' mammary. α-Lactalbumin 100x.
A; Bright field. B; Dark field. In dark field, the image is reversed so that expression is seen as white dots. This allows greater sensitivity.

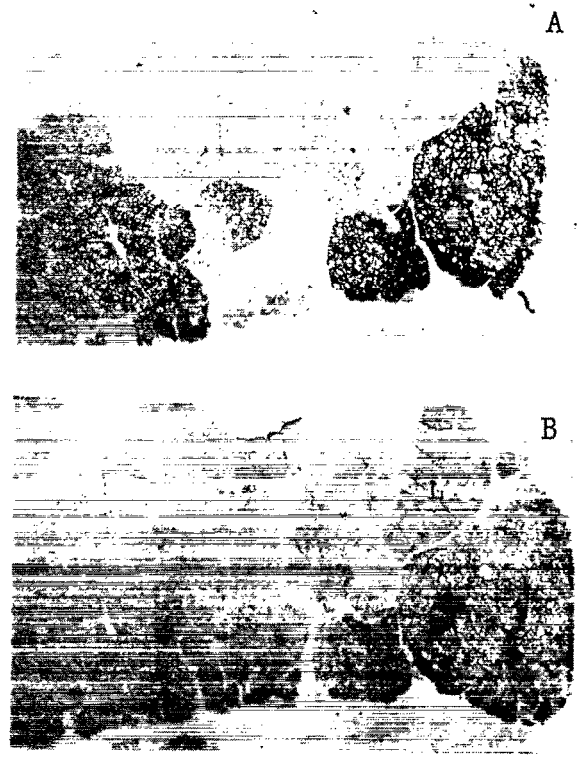

FIG 3 Lactating mammary, α-lactalbumin 10x. A; Signal. B; Negative control (labeled sense RNA).
At this lower magnification, the dots representing mRNA expression coalesce to form black areas over the cells.

In the 'pregnant' mammary gland (Fig. 2) distinct areas of α-lactalbumin were seen. These are distributed in a seemingly random fashion. Histologically it was difficult to see a difference between expressing and non expressing cells and alveoli. Darkfield reverses the image, making black become white so that it is easier to see the white areas of expression and that there was a wide range of expression levels. In some areas, clusters and regions of whole alveolar lobules, in others, just single cells expressed. Although the mammary gland undergoes rapid growth and differentiation prior to parturition, it is unlikely that cellular replication is the cause of variation in expression, as lactating cells can enter mitosis. Traurig (1967) reported that ^{3}H thymidine is incorporated in secreting cells. and Hollmann noted that mitotic figures are observed with the electron microscope in lactating cells with a highly organized cytoplasm.

The low magnification (Fig. 3) of a section from a newly lactating gland demonstrates strict sectoring of α-lactalbumin expression. Similar populations of secretory cells expressed at similar levels. Higher magnification (not shown) revealed that the central area of tissue apparently consisted of alveoli that were characterized by the presence of fat globules in abundance in the apical surface of the cells and in the lumen. Some alveoli had thick and others, thin walls. The latter were larger and had a copious amount of lumenal material. In contrast to the situation in the 'pregnant' gland, no expression was seen in this area. On the peripheries of this section, another type of alveoli was present. These were generally bigger, and contained less lumen, although this may have been lost during processing. The latter were producing a large amount of α-lactalbumin mRNA but even at this low

magnification, an inverse relationship between size and expression was apparent. The highest level of expression was found in thick-walled (collapsed) alveoli, having a moderate amount of stromal material underlying the epithelium. A slightly lower level of expression was seen in the distended thin-walled alveoli with homogeneous contents. The latter alveoli were essentially a double layer of secretory cells separated by a basement membrane and the odd myoepithelial cell. The difference in the two expression levels was possibly due to increased cell density, but it may be that the 'collapsed' alveoli had been recently drained of milk and consequently were initiating more synthesis. These alveoli, with more columnar type cells and reduced lumenal volume, are characteristic of 'filling' alveoli (Mayer and Klein, 1961). A very rapid local feedback may be operating here, controlling the rate of milk production.

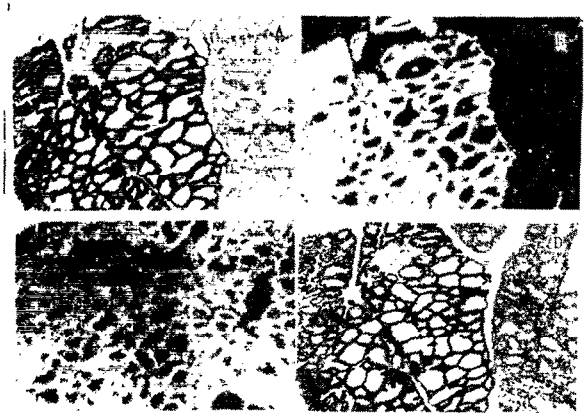

FIG 4 Serial sections of lactating mammary. The fat globule containing alveoli are on the right third of each photo. α-Lactalbumin signal 100x. A; Bright field. B; Dark field. C; β-Actin signal, dark field. D; Unprobed, stained with haematoxylin and eosin.

Many of the histological features mentioned above are illustrated in Figure 4. One can see that, in contrast to the α-lactalbumin expression shown in Fig. 4A, actin expression was higher in the fat globule containing alveoli, than in the non fat globule containing alveoli. This indicates that although the former cells were not making α-lactalbumin, they were still active. Disruption of cytoskeletal elements, of which actin is a part, occurs in both secretion, non secretion (Mayer and Klein, 1961; Nickerson and Akers, 1984) and involution (Hurley 1989). Salazar and Tobo (1974) in their paper show a photomicrograph with very similar histology to those shown above which they explain in terms of resting and lactogenic lobules. Hollmann (1974) agrees that they may be alternating between the two states.

In mice, β-casein production (day 12 onwards, Mina Bissell, personal communication) and α-lactalbumin secretion (Cowie and Tindal, 1971) are seen in virtually all mammary epithelial cells. At this stage of lactation, the mammary gland of mice is likely to be under a greater relative milk demand than in the sheep studied here, and involuting epithelia are rapidly sloughed off in rodents whereas this is not significant in bovines and other ruminants (Hurley, 1989). This may account for the different pattern of expression.

CONCLUSIONS

The observations of areas with heterogenic levels α-lactalbumin expression and of their compartmentalisation from those areas containing fat are intriguing and suggest a number of possible hypotheses.

First, that if all lactating cells produce all the components of milk then synthesis and secretion of α-lactalbumin is closely related to the long term activity of the cell. Those 'non-messenger' RNA containing alveoli with an abundance of fat therefore, are either resting or regressing. Classical histological studies indicate that the size of the fat globules and degree of distension of the alveoli are characteristic of cells in the early stages of regression (2 to 14 days) (Mayer and Klein, 1961; Hurley, 1989; Holst *et al.*, 1987). Since macrophages are absent from these alveoli it is likely that they are resting rather than regressing. Of great interest was that histologically similar alveoli in discrete and separate lobules showed similar levels of α-lactalbumin expression. The fact that adjacent lobules were apparent with high and zero levels of expression respectively emphasizes the exquisitely local control over mammary gene expression and its possible connection with to localized milk stasis.

Second, the variation of expression seen among α-lactalbumin mRNA containing alveoli suggests that synthesis of this RNA is somehow involved in the immediate regulation of total milk protein synthesis and

volume, ie, those alveoli that are full or have just been milked are expressing less α-lactalbumin than those which are 'filling up' (the collapsed alveoli). Conversely, overall protein production including that of α-lactalbumin may be reduced in the distended gland, possibly by an inhibitor such as that isolated by Wilde (personal communication).

Lastly, and in conflict with the current dogma, an alternative explanation is that in the lactating gland, synthesis of this mRNA (possibly the α-lactalbumin protein itself) may be compartmentalized and found in epithelial cells that synthesize less fat. Further studies with genes for fat synthesis and immunohistochemical probes for α-lactalbumin may clarify this. (Lee *et al.*; 1984)

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