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Evidence for a diffusible factor influencing the switching of ovine and bovine milk gene expression leads to identification of factors potentially involved

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ABSTRACT

Our earlier in situ hybridisation studies have shown heterogeneous expression of milk protein genes. At some sites, a sharp demarcation of mRNA expression is seen at the basement membrane between 'active' and 'inactive' tissue, while at other nearby sites, a reciprocal gradient of mRNA expression about 10 cells wide is seen. The alveoli in these latter 'inactive' sites contain leucocytes which indicates that they have been 'inactive' longer than the former. This suggests a factor could be diffusing between 'inactive' and 'active' tissues and influencing milk gene expression. We used a consensus oligomer corresponding to the linkers between the fingers of zinc finger DNA binding proteins in a polymerase chain reaction performed on cDNA from 'active' versus 'inactive' mammary mRNA. Differences and similarities were observed in the sizes of the DNA products from each sample. Several of the bands present in the 'active' cDNA and absent from the 'inactive' cDNA were cloned and some were found to have homologies to zinc finger and other DNA binding proteins. It is possible that we have partially isolated a component of the pathway that maintains lactation.

Keywords: in situ hybridisation; milk gene expression; zinc finger; mammary.

INTRODUCTION

Previous in situ hybridisation studies from our laboratory have shown that expression of certain milk protein genes is very high in most parts of the mammary glands of sheep and cattle, while in other areas apparently in stasis, there is almost none (Molenaar et al., 1992). Conversely, expression of lactoferrin, a marker of involution, was high in these areas of stasis and low in areas having an active secretory appearance.

In some examples, the demarcation between the expression of one set of genes and the non expression of the other was exactly matched with the cell type and a sharp transition of gene expression occurred at the basement membrane between the two cell types. In other examples however, narrow, opposing gradients of gene expression were seen that appeared to originate from the alveoli at rest and extend into the active regions.

Whether the switch in gene expression was sharp or a gradient, appeared to depend on the length of time the 'resting' alveoli had been in this state. This pattern suggests that a factor controlling the switching of milk gene expression was diffusing or being transported from one type of alveoli to the other.

The identification of genes involved in the synthesis of this factor may suggest other approaches to the pharmacological or genetic manipulation of milk production. To influence gene expression at the DNA level, a molecule must have a DNA binding activity. Many such molecules contain parts that are shaped like fingers so they can 'grip' the DNA and also have zinc ions located within the fingers, hence the name. Assuming that such a molecule was a likely candidate, we used a short zinc finger consensus oligonucleotide designed by Hoovers (Hoovers et al., 1992) and a 20 base dT oligonucleotide as a primer pair in a polymerase chain reaction performed on cDNA from active versus involuting mammary tissue in the same udder. The polymerase chain reaction (PCR) is a technique which exponentially 'photocopies' specific nucleic acid sequences and so is extremely powerful and sensitive. Both similarities and differences were observed in the DNA band sizes obtained. We have cloned and sequenced some of these DNA products and searched for homology to all other known genes.

METHODS

In Situ Hybridisation. Representative samples were taken from the udders of three 10 week lactating ewes and mounted onto glass slides. Serial sections were probed as previously described (Molenaar et al., 1992) with bovine α-lactalbumin (Hurley and Schuler, 1987), α-S1 casein (Stewart et al., 1984), butyrophilin (Jack and Mather, 1990) and lactoferrin (Mead and Tweedie, 1990) 35S-UTP labeled cDNA riboprobes at moderate stringency, washed, then exposed under Ilford K5 emulsion diluted 1/1 for 1-6 weeks as appropriate and stained with Haematoxylin and Eosin.

Fortuitously, at the time of slaughter, one dairy cow was found to have 3 active quarters and one undergoing involution (determined by histology and by hybridisation with α-lactalbumin and lactoferrin riboprobes). Polymerase chain reactions (PCR) were performed on cDNA was made from mRNA obtained from an active quarter and of the involuting

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quarter, from this animal's udder. PCR was performed four times with slightly varying cycling conditions on the cDNA according to the method of Frohman (Frohman, 1990) in Jeffrey's PCR buffer (Jeffreys et al., 1988) with 100x excess of the primers CAY ACW GGD GAR AAR CCH TA and dT(17). The PCR products were separated and visualized by agarose gel electrophoresis.

Cloning and analysis. Bands were selected from the ‘active’ cDNA PCR products that were not present in the ‘inactive’ cDNA, cut from the gels, purified and ligated to either pGEM3zf+ (Promega) or pDK101 (Kovalic et al., 1991). Transformants were screened by agarose gel electrophoresis and restriction digestion analysis. Plasmids containing inserts were sequenced using PCR according to Wang et al. and at the automated DNA sequencing facility at Auckland University. The sequences were compared to those residing in the National Center for Biotechnology Information, NIH, USA, using the programs Blastn and Blastx on the BLAST electronic network service. Blastn compares DNA sequences and Blastx, which is more sensitive, converts the sequence to protein and compares it to protein databases.

RESULTS

In Situ Hybridisation. As observed previously, in mammary glands of sheep and cattle, the majority of a gland in active lactation has an active secretory appearance. In situ hybridisation studies showed high expression of α-lactalbumin and α-S1 casein mRNAs in these areas and low or absent expression in other areas containing numerous fat droplets, which appeared in stasis (Fig 1a & b). Conversely, expression of lactoferrin, a marker of involution, was high in those latter areas of stasis, and low in the secretory areas. In some examples, the demarcation between the expression of one set of genes and the non expression of the other was exactly matched with the cell type and a sharp transition of gene expression occurred at the basement membrane between the two cell types (Fig1 a & b). In other examples however, opposing gradients of gene expression were seen that appear to originate from the alveoli at rest and extend into an 10-20 cell thick zone of cells in the active regions (Fig1 c-f).

PCR. In each PCR reaction pair, several products of similar size were observed. One or more products were also observed that were unique to the PCR cDNA from the ‘active’ tissue. One ~200 bp band in particular was present in 3/4 PCR reactions (Fig a, b, d). Clones originating from bands a(26, 29, 74)(Fig a), b(12, 18) (fig c), and c(57)(Fig c) have been identified. Clones 18 and 29, a concatamer, have high homologies (>75%) to lactoferrin. Blastx found homologies of clone 12 with 54 protein fragments, 3 of which were DNA binding proteins of some kind. Similarly, 35 homologies were found with clone 26, 13 of which were zinc finger proteins. BlastN found that clone 57 had 90% homology over a 100 bp sequence to exon 15 of human platelet glycoprotein IIIa, and BlastX found homologies to 19 protein fragments, with the greatest homology to a salmonella lipopolysaccharide 1,2-n-acetylglucosaminetransferase. BlastX found that clone 74 had homology to 39 gene fragments, 2 of which were zinc finger proteins while BlastN found 26 homologies to gene fragments most of which were to a line-1 element but did include a human zinc finger gene. The translated consensus oligomer, HTGEKP, was a homologous component present in many of the sequences found.
FIGURE 2: PCR analysis of cDNA prepared from 'active' and 'inactive' mammary total mRNA. Small letters denote bands cloned.

2A. PCR1. Lanes from left to right: 1; λ HindIII/EcoRI marker (λH/E). 2; Active cDNA (AcD). 3; Inactive cDNA (IcD). 4; Blank. 5; pGem3/HindIII marker (pG/H). 1% agarose.

2B. PCR2. Lane: 1; λH/E. 2; AcD. 3; IcD. 4; No cDNA control. 5; pG/H. 3% agarose.

2C. PCR3. Lane: 1; U-l/E. 2; AcD. 3; IcD. 4; pBR322/HindIII (p322/H). 3% agarose.

2D. PCR4. Lane: 1; p322/H. 2; AcD. 3; IcD. 1% agarose.

2E. Restriction analysis of some insert containing clones. Lane: 1; λH/E. 2; clone 12. 3; clone 18. 4; clone 26. 5; clone 20. 6; clone 37. 7; clone 74. 8; pG/H. p322/H. Marker sizes are from top to bottom, λ H/E in Kbp: 21, 5.1, 4.2, 3.5, 2.0, 1.9, 1.6, 1.4, 0.95, 0.83, 0.56, pG/H in bp: 1198, 517, 396, 350, 152, p322/H in bp: 1632, (517, 506), 396, 344, 298.
DISCUSSION

The presence of leucocytes in the 'inactive' tissue shown indicates that the tissue had been in that state for at least several days because that is the infiltration time (Molenaar et al., 1994). The association of leucocytes with the reciprocal gradient and not with the sharp gradient suggests that there is something different about the two examples of ‘inactive’ tissue shown even though they were located within 10 mm of each other in the same mammary gland. The gradual increase in lactoferrin and decrease in casein and a-lactalbumin mRNA expression in the ‘active’ tissue at the ‘active/inactive’ boundary seemed to indicate that some factor in the ‘inactive’ tissue was influencing gene expression in the ‘active’ tissue. A DNA binding protein must be involved at some point in the pathway controlling gene expression. Hoovers has successfully used an oligonucleotide specific for the ‘link’ between contiguous zinc fingers to isolate zinc finger protein genes from cosmids obtained from mouse-human cell lines enriched for chromosome 11p (Hoovers et al., 1992). We felt that the same oligomer should function in a PCR reaction. As the figures show, we were able to display different mRNA ‘fingerprints’ from ‘active’ and ‘inactive’ mammary tissue from the same genetic background and clone some of these fragments.

Several of the clones derived from the ‘active’ tissue are highly homologous to lactoferrin. Actively lactating dairy mammary tissue does express small but detectable amounts of lactoferrin mRNA (Molenaar et al., 1994) When the linker sequence was compared to cow lactoferrin using the Pustel DNA matrix within the DNA sequence analysis computer program MacVector (Kodak Life Science Products, USA), 31 segments were found having more than 65% homology to the degenerate oligomer at various points along the sequence. It is likely that many of these sites can be primed under the PCR conditions used and so result in the formation of PCR products. Indeed, this is the probable explanation for the background smear seen in the gel lanes. Given the sensitivity of the PCR reaction, we were lucky that these lactoferrin PCR products did not overwhelm the screening.

The reciprocal gradient of gene expression seen in the in situ hybridisation of the sheep mammary gland is significant as it shows that some factor does act at a local or cell to cell level. It is not too long step to extrapolate that a similar mechanism would occur in the bovine but it is not easy to macroscopically find such local variations in fresh mammary tissue. However it is possible to macroscopically see a difference between active and involutingudder, and to subsequently confirm this. We could be confident that among those populations of cells, similar mechanisms would be operating as seen in the sheep. By comparing mRNA from such tissues in the same dairy animal, we were able to eliminate the risk of animal to animal variation, and be able to study a system with more relevance to the industry.

The observation that the gradient appeared to originate from the inactive fraction of the mammary gland lead us to expect that PCR with the linker consensus oligomer would find mRNA unique to the inactive fraction of the gland. These putative mRNA’s would possibly have a role in shutting down lactation. The opposite finding, however, is very exciting because the unique cDNA PCR products were all derived from the ‘active’ fraction of the gland and found to code for potential DNA binding proteins. These proteins may well have a role in maintaining lactation, a discovery of greater interest to the dairy industry.

Further work is required to further characterise these cDNA fragments by northern, Southern, and in situ analysis to verify if they are biologically important and to clone the genes. It remains to be seen whether their mRNA’s are expressed in similar patterns as the genes shown.

SUMMARY

We have isolated fragments of cDNA’s which code for transcriptionally active DNA binding proteins and may be part of the ‘on’ switch for lactogenesis.

REFERENCES


