BRIEF COMMUNICATION

The use of cDNA microarrays to investigate changes in gene expression in the involuting bovine mammary gland

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The molecular mechanisms and pathways involved in involution of the udder and which coordinate and regulate the processes of cell death and proliferation after weaning/cessation of milking in ruminants, are poorly understood. The aim of this study was to use cDNA microarray analysis to identify genes and pathways involved in involution of the bovine mammary gland. Ultimately, because this process underpins lactation traits such as persistency and mammary responses to milking frequency (Stelwagen, 2001; Vetharaniam *et al*., 2003), this information may allow the development of novel strategies for improving milk production especially in the later stages of lactation.

Involution of the bovine mammary gland was induced by abrupt termination of milking in 48 non-pregnant Friesian dairy cows at mid-lactation (average days in milk, 92 ± 3). The cows were in their first lactation with an average daily milk yield of 14.3 ± 0.3 kg/cow. Somatic cell count at the start of the experiment was 1.96 ± 0.29 x 10^3 cells/ml. Alveolar mammary tissue were obtained following slaughter at 0, 6, 12, 18, 24, 36, 72 and 192 h (n=6 per group) after the last milking. Animals were slaughtered by captive bolt and exsanguination and samples (approximately 30 g) of secretory alveolar mammary tissue were obtained from the middle of the upper one-third of the gland of the rear quarter and snap-frozen in liquid nitrogen. The tissue was ground and total RNA was extracted from an aliquot of tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

Microarray analysis comparing the 6 and 36 h time points following the last milking was carried out. The 6 h post milking time point was used for the microarray comparisons because it is the most representative of actively lactating mammary glands. All the time points were used in real-time PCR analysis for verification of microarray analysis. AgResearch has developed a bovine Expressed Sequence Tag (EST) database which incorporates 203 k ESTs, derived from 50+ tissue libraries and ~40 k contiguous sequences. ESTs were selected for arraying from the library to provide representation from a wide range of tissues but favoured mammary gland specific ESTs and included some novel sequences. Each slide had specific 23 k ESTs arrayed representing low redundancy 16550 ESTs with known mRNA SwissProt hits and 6772 with no hits, selected from mammary and a variety of other bovine libraries, plus controls.

Total RNA was isolated from tissues (6 and 36 h time points) using TRIzol (Invitrogen), labelled with Cy3 and Cy5 dyes and hybridised to microarray slides as described by the manufacturer (Ambion). Microarray slides (n=24) were hybridised in a daisy chain design. The slides were scanned using a Packard 5000i scanner (GSI Luminomics). The images were analysed and data generated using GenePix Pro 3.0. Each cow at each time point was compared with 2 cows from the opposite time point along with its dye reversal slide. For each slide, log ratio data was normalised using a mixed model with spatial autocorrelation using REML in GenStat. The ESTs were classified into functional groups using a web-based application, DAVID (Dennis Jr., 2003). This application enabled annotation based on Gene Ontology organising principles, which grouped the genes based on their biological process, thus providing information on the possible role of genes and functional groups of interest during mammary involution.

Several milk proteins; α-lactalbumin, αS1-casein and lactoferrin, were selected for verification of the microarray results. Total RNA was extracted from tissue (0, 6, 12, 18, 24, 36, 72 and 192 h (n=6/group)), and converted to cDNA using a SuperScript II First-Strand Synthesis system as described by manufacturer (Invitrogen). Quantitative real-time PCR analysis was carried out using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Detection of the product was by SYBR Green I, using the Universal PCR Master Mix (Applied Biosystems, Sequence Detection System, Chemistry Guide, 2003). For each assay two control reactions were included; a reverse transcriptase-negative control, and omission of the template (no template control). Any amplification occurring in these control reactions would indicate the presence of non-RNA template. The thermal cycling program was 95 °C for 10 min followed by 45 cycles of 95 °C for 15 sec, 58 °C (α-lactalbumin and αS1-casein) or 60 °C (lactoferrin) for 30 sec, 72 °C for 30 sec, 78 °C for 10 sec. Dissociation curve analysis confirmed that only 1 product was amplified. The amplified products were verified by sequencing and on

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2% agarose gels. The threshold cycle (C_T) for each gene and the internal control β-actin was generated by real-time PCR and used to quantify the relative abundance of each gene (Applied Biosystems, Sequence Detection System, Chemistry Guide, 2003). Differences between means at each time point were analysed using analysis of variance in Minitab (Minitab Release 13 for Windows, 2000). The least significant differences identify the means significantly different from each other ($P<0.05$). Results were expressed as the fold change relative to the 6 h mean and reported as mean ± the standard error of the mean (SEM).

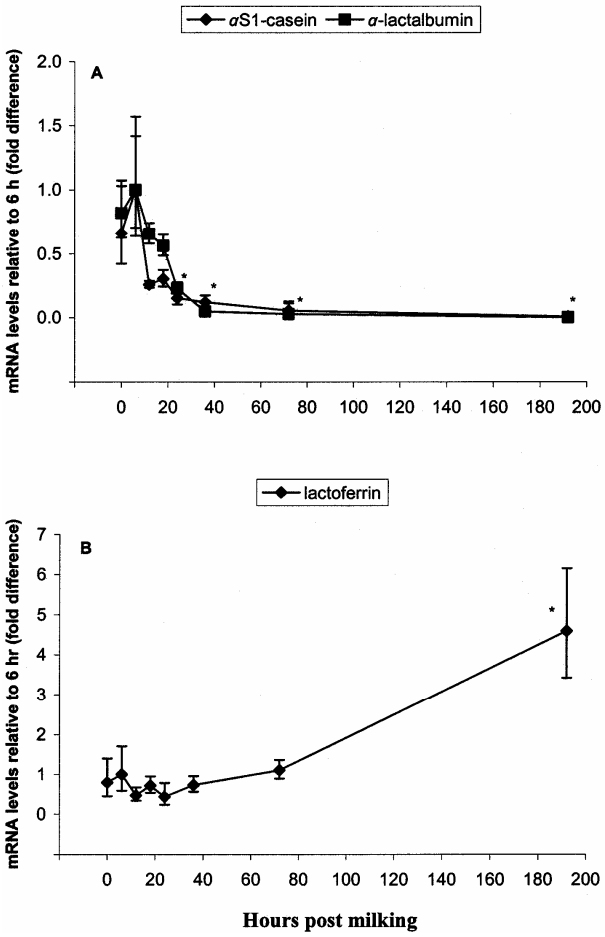
The microarray results showed changes in expression of 5300 ESTs ($P<0.01$) and 3300 ESTs ($P<0.001$) at 36 h compared to 6 h post-milking. At $P<0.01$, 1336 ESTs changed expression by at least 1.5-fold; at $P<0.001$ the comparable figure was 1184. For ESTs associated with cellular processes of cell communication, death, differentiation motility, growth and/or maintenance there were 359 ESTs up-regulated and 253 down-regulated, by at least 1.5-fold at 36 h compared to 6 h post-milking. Of the ESTs involved in physiological processes, which include metabolic pathways, there were 199 up-regulated and 198 down-regulated by at least 1.5-fold at 36 h post-milking. The metabolic pathways having the most ESTs differentially expressed by at least 1.5-fold were; biosynthesis (51 ESTs up-regulated and 20 down-regulated), catabolism (21 ESTs up-regulated and 19 down-regulated) and nucleic acid metabolism (46 ESTs up-regulated and 51 down-regulated). There were 54 ESTs up-regulated and 22 down-regulated at 36 h post-milking by at least 1.5-fold that are involved in development. There were 573 ESTs up-regulated and 533 down-regulated by 1.5-fold ($P<0.01$) which were not classified by the programme DAVID. In total there were 1220 ESTs which were up-regulated by at least 1.5-fold and 1027 down-regulated at 36 h compared to 6 h post-milking.

The 25 ESTs for αS1-casein detected in the micorarray analysis showed an average down-regulation by 1.43 ± 0.11 fold, at 36 h relative to 6 h post-milking. The 6 ESTs detected for α-lactalbumin showed an average down-regulation by 1.71 ± 0.22-fold, and the 23 lactoferrin ESTs detected in the microarray analysis showed an average up-regulation of 1.59 ± 0.097-fold, at 36 h relative to 6 h post milking.

In comparison to the microarray results, quantitative real-time PCR analysis showed that at 36 h, relative to 6 h following the last milking, α-lactalbumin mRNA levels were dramatically decreased (20-fold), αS1-casein mRNA levels were decreased by 8.3-fold (see Figure 1A) and lactoferrin mRNA levels were unchanged at 36 hr to 6 h post-milking (see Figure 1B).

The αS1-casein mRNA levels tended to be lower ($P<0.1$) by 12 h and were 6.6-fold decreased ($P<0.05$) by 24 h relative to 6h post-milking (see Figure 1A). The α-lactalbumin mRNA levels were 4.4-fold decreased by 24 h ($P<0.05$) relative to 6 h post-milking (see Figure 1A). The expression of both αS1-casein and α-lactalbumin continued to decrease ($P<0.05$) to 8 d after last milking (see Figure 1A). In contrast, the lactoferrin mRNA levels were increased at 8 d ($P<0.05$, 4.6-fold) relative to 6 h following the last milking (see Figure 1B).

FIGURE 1: Changes in mRNA levels of αS1-casein, α-lactalbumin and lactoferrin, during involution in mammary alveolar tissue of lactating cows (n=6 per time point). Data are expressed as mean ± SEM and P values (* $P<0.05$) are relative to 6 h time point for respective genes.



This study described changes in gene expression in the bovine mammary gland. The microarray analysis has provided evidence of widespread changes in bovine mammary gene expression occurring within 36 h post milking. Many of the genes that were differentially expressed at 36 h post milking were involved in cellular processes such as cell death and growth and/or maintenance. Genes involved in metabolic functions that were differentially expressed were mostly from both biosynthetic and catabolic pathways, as well as nucleic acid metabolism. Our results demonstrate that the changes in mRNA levels for the milk proteins αS1-casein and α-lactalbumin in the involuting mammary gland of ruminants is very rapid, occurring as early as 24 h. Previous studies in ruminants have shown that three days following the termination of milking, casein and α-lactalbumin mRNA levels were reduced but to a lesser degree than in rodents (Goodman & Schanbacher, 1991). In lactating rodents, when involution was induced by

litter removal, the mRNA levels of α-, β- and γ-casein decrease within 24 h by up to 95% (Travers *et al.*, 1996). This suggests that the response to induced involution in ruminants is slower than in rodents. Previously, we have reported low concentrations of lactoferrin in milk during established lactation which is increased following mammary engorgement induced by once-daily milking (Farr *et al.,* 2002). Lactoferrin mRNA levels are also increased during mammary involution in engorged, secretory alveoli (Molenaar *et al.,* 1996). Our results demonstrate lactoferrin mRNA levels in mammary alveoli are increased as early as 8 days following the last milking.

The microarray results show genes which are differentially expressed at 36 h post milking compared to 6 h are within 1.5- to 4-fold changes in expression. Previous DNA microarray experiments have reported similar differences in expression (Der *et al*., 1998; Chang *et al.*, 2000; Zhu *et al*., 1998). Considering the level of changes in gene expression and array hybridisation intensity, validation of differentially expressed genes identified by microarray analysis is required (Rajeevan *et al.,* 2001). Our results show the level of gene expression can be different between the cDNA microarray and the sensitive real-time RT-PCR analysis. This is in agreement with previous DNA microarray experiments which suggest the true expression differences for specific members of gene families may be masked by cross-hybridisation in microarrays (Rajeevan *et al.,* 2001).

Our data have shown that many gene pathways are both activated and inactivated during early involution. Future research will focus on key pathways involved in apoptosis in order to devise intervention strategies that may delay gradual involution and increase lactational persistency.

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