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BRIEF COMMUNICATION: DNA methylation events associated with the suppression of milk protein gene expression during involution of the bovine mammary gland

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INTRODUCTION

During lactation, prolactin activates STAT5 (Signal Transducer and Activator of Transcription), which binds to conserved DNA sequence motifs in promoters of milk protein genes to stimulate their expression (Liu *et al.,* 1997). At drying-off, mammary involution is characterised by a rapid decrease in milk protein gene expression (Goodman & Schanbacher, 1991), including αS1-casein (Singh *et al.,* 2008). The αS1-casein promoter features a doublet STAT5 binding site containing 3 CpG dinucleotides. During lactation, these CpG dinucleotides are hypomethylated but become methylated following *Escherichia coli* infection of the gland. This results in chromatin condensation and is associated with an acute shutdown of αS1-casein synthesis (Vanselow *et al.*, 2006). To understand the regulation of milk production at the molecular level, this study aimed to investigate the role of DNA methylation in giving rise to an epigenetic effect of silencing milk protein gene expression during bovine mammary gland involution.

MATERIALS AND METHODS

Mammary involution was induced by abrupt termination of milking in 42 non-pregnant Friesian heifers at mid-lactation after 89.1 ± 2.2 days in milk. Alveolar mammary tissue was obtained following slaughter at 0, 6, 12, 18, 24, 36, 72 h (n = 6 per group) after the last milking. Further samples were collected in the following season from 10 similar heifers slaughtered at 72 (n = 4) or 192 h (n = 6) following the last milking after 116.9 ± 6.0 days in milk. Prior to cessation of milking, the daily milk yield was 14.3 ± 0.3 kg/cow and somatic cell count was 165 ± 30 x 10^3 cells/mL. A liver sample was collected as a positive control from a lactating cow.

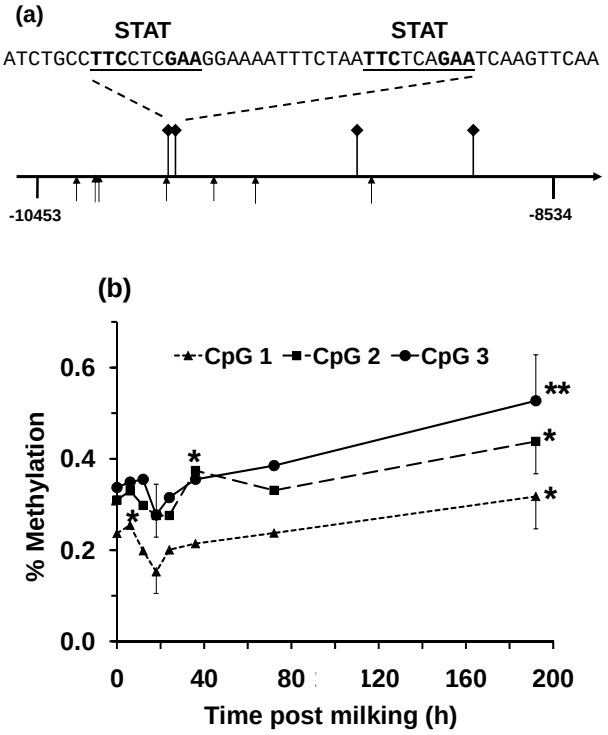
Genomic DNA was isolated from the tissue (Qiagen, Valencia, CA, USA) and bisulfite treated (Zymo Research, Orange, CA, USA), as described by the manufacturer. Following polymerase chain reaction amplification, the products were confirmed on 1.5 % agarose gels and prepared for quantitative methylation analysis, as described by the manufacturer (http://www.sequenom.com). Samples were subjected to Sequenom MassARRAY analysis (Ehrich *et al.*, 2005). DNA methylation analysis of the 3 CpG dinucleotides around the distal STAT5 binding site (~ -10 kbp) in the αS1-casein promoter was carried out. The resultant spectra's methylation ratios were generated by the Epityper software v1.0 (Sequenom). The percentage of methylation at each CpG site was analysed with a linear model fitting year and hours post-milking using GenStat (Payne *et al.*, 2007). Data were expressed as mean percentage methylation for each hour adjusted for year.

RESULTS AND DISCUSSION

The genomic map (Figure 1a) shows the position of the 3 CpG dinucleotides which are in close proximity to the functional STAT5 binding site in the distal αS1-casein promoter (modified from Vanselow *et al.*, 2006). During lactation (6 h post-milking), the percentage of methylated DNA molecules at CpG-1, -2 and -3 was 25, 33, and 35%, respectively (Figure 1b). By 18 h post-milking, the methylation level of CpG-1 had decreased to 15% ($P < 0.05$), compared with 6 h, and both CpG-2 and -3 methylation levels were 28%. By 192 h post-milking, the degree of methylation of CpG-1, -2 and -3 was increased to 32% ($P < 0.05$), 44% ($P < 0.05$) and 53% ($P < 0.01$), respectively, compared with 18 h (Figure 1b). CpG-2 methylation levels were increased as early as 36 h (37%, $P < 0.05$), compared with 18 h post-milking.

In the present study the level of methylation in the fully lactating gland, 6 h post-milking, is three times higher than that previously reported by Vanselow *et al.*, 2006. This may be partly due to differences between a pasture and grain diet, and/or milk yield. The decline in methylation at 18 h post-milking compared with 6 h may be explained by the multiple physiological events which occur at 16 to 18 h in response to the cessation of milk removal in dairy cows (Davis *et al*., 1999). These events occur prior to the decline in prolactin-STAT5 signalling (Singh *et al.*, 2009) and milk protein gene expression that occurs at 24 h post-milking (Singh *et al.*, 2008). The αS1-casein mRNA levels continue to decline to 192 h post-milking (Singh *et al.*, 2008). Compared with 6 h post-milking, an increase in methylation was observed only when the αS1-casein mRNA levels were decreased by over 120-fold at 192 h post-milking. In addition, at 192 h post-

FIGURE 1: (a) Genomic map around the distal aS1-casein promoter (modified from Vanselow *et al.*, 2006). STAT5 binding sites (lines with carets) between -10453 and -8534 in the αS1-casein promoter, showing the functional doublet STAT5 binding site. Arrows show the position of CpG dinucleotides. CpG 1-3 were analysed for methylation status during involution. (b) Time course of changes in mean % methylation of CpG 1-3 sites in close proximity to the αS1-casein promoter. Time points 0 h to 24 h and 192 h, n = 5; 36 h, n = 6, 72 h, n = 9. Error bars show standard error of difference (below lines) with significance relative to 18 h time point. The standard error of difference (above lines) is shown at 18 h for comparing with time points up to 24 h and at 192 h for comparing 192 h with each time point up to 24 h.



milking, there was a dramatic decline in αS1-casein protein levels (K. Singh, Unpublished data) compared with all the earlier time points. The methylation level at 192 h post-milking for the 3 CpG dinucleotides (mean value, 43%) was similar to the mean values reported previously for mammary tissue collected from pubertal (42%), non-lactating (36%) and mastitic mammary tissue (47%) (Vanselow *et al.* 2006). In agreement with our results, the αS1-casein protein levels were barely detectable when the 3 CpG sites were methylated to this degree. Our results indicated that during involution, the decline in prolactin signalling was associated with a decline in milk protein mRNA (Singh *et al.*, 2009). In contrast, during *E. coli* infection of the udder, the systemic levels of prolactin remained unchanged compared to uninfected controls (Vanselow *et al.,* 2006). During involution, although DNA methylation may not initiate the decline in αS1-casein mRNA, the degree of DNA methylation of the 3 CpG sites plays a role in silencing αS1-casein expression in both involution and infection of the udder.

Although milk protein mRNA and protein levels are low in alveolar tissue by 192 h post-milking (Singh *et al.*, 2008), previous reports have demonstrated that milk production can be fully restored after 7 days of non-milking (Dalley and Davis, 2006). This indicates that in the present study, the mammary gland was still in the early stages of involution and that the irreversible phase had not yet been reached. It is possible that methylation levels continue to increase as the mammary gland becomes more involuted. The methylation levels at 192 h post-milking for CpG-1 and -2 were not as high as in the liver (CpG-1, -2, and -3 were 57%, 63%, and 58%, respectively) which does not express αS1-casein. These values are similar to those previously reported, where 67% of the DNA molecules were methylated in the liver of a lactating cow (Vanselow *et al*., 2006).

Future research will focus on changes in methylation status of other major milk protein genes during involution. Understanding the role of epigenetic mechanisms in regulating milk production may result in novel approaches and/or technologies for enhancing the lifetime lactation performance of dairy cows through manipulating epigenetic mechanisms.

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