DNA-remethylation around a STAT5-binding enhancer in the α S1-casein promoter is associated with abrupt shutdown of α S1-casein synthesis during acute mastitis

Jens Vanselow, Wei Yang, Jens Herrmann, Holm Zerbe¹, Hans-Joachim Schuberth², Wolfram Petzl², Wolfgang Tomek and Hans-Martin Seyfert

Research Institute for the Biology of Farm Animals (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

¹Veterinary Faculty, Ludwig-Maximilians-University, Munich, Sonnenstrasse 16, 85764 Oberschleissheim, Germany

²Institute of Immunology, University of Veterinary Medicine, Bischofsholer Damm 15, 30173 Hannover, Germany

(Requests for offprints should be addressed to H-M Seyfert; Email: seyfert@fbn-dummerstorf.de)

J Herrmann is now at RWTH, Institute for Clinical Chemistry and Pathobiochemistry, Pauwelsstr 30, 52074 Aachen, Germany

Abstract

Prolactin stimulates the expression of milk genes during lactation through the activation of STAT5 transcription factors, which subsequently bind to their cognate target sequence on the promoters. Demethylation of 5methylCpG dinucleotides permits the tissue-specific accessibility of transcription factor-binding sites during development, but remethylation has not been shown to contribute to acute suppression of gene expression. We characterize functionally a novel STAT5-binding lactational enhancer in the far upstream promoter (~ -10 kbp) of the bovine α S1-casein-encoding gene. This promoter area is hypomethylated in the lactating udder only. Remethylation of this area accompanies an experimentally elicited acute shutdown of casein synthesis in fully lactating cows, whose udder quarters have experimentally been infected with a pathogenic *E. coli* strain. Within 24 h after infection, the relevant promoter area was remethylated from 10% of the DNA molecules in the uninfected control quarters to $\sim 50\%$ in the infected quarters, the typical values for fully lactating and not lactating udders respectively. Increased methylation resulted in tighter chromatin packing. Concomitantly, the α S1-casein mRNA concentration dropped to $\sim 50\%$ while the protein synthesis was shut down to $\sim 2\text{--}5\%$ in the infected quarters, alone. The methylation status of the promoter from a not lactationally regulated gene was unaltered, and the distal α S1-casein promoter was not remethylated in udder quarters with subclinical *Staphylococcus aureus* infections featuring sustained casein synthesis. Hence, infection-related remethylation of the α S1-casein promoter and chromatin remodelling serves as an acute, spatially restricted regulatory mechanism, which might insulate the promoter against the systemically unchanged high levels of circulating prolactin. This provides a rare example for an acute regulatory significance of CpG methylation.

Journal of Molecular Endocrinology (2006) **37**, 463–477

Introduction

α S1-Casein is the most abundantly expressed milk protein in the cow (Brunner 1981), expressed only during lactation in mammary epithelial cells (MEC) lining the alveoli of the milk parenchyma of the udder. The most prominent signal to orchestrate the expression of the lactation-specific genes is the hormone prolactin. It is indispensable for mammary gland development and function (Vonderhaar 1988, Vonderhaar & Ziska 1989) and elicits casein expression in synergy with glucocorticoid hormones *in vivo* (Guyette *et al.* 1979, Rosen *et al.* 1980) and in MEC models (Doppler *et al.* 1989, Lechner *et al.* 1997). The cascade of factors and events transducing the prolactin signal via the JAK2/STAT5 pathway into the cell and resulting in stimulated expression of target genes has been well established during the last decade (Hennighausen *et al.* 1997, Yu-Lee *et al.* 1998, Groner

2002). Ultimately, the cascade activates in the cytoplasm the closely related (Seyfert *et al.* 2000) STAT5A and STAT5B transcription factors. After translocation into the nucleus, they bind to a conserved DNA sequence motif in target promoters, known as GAS sequence (TTCNNNGAA; Schindler *et al.* 1995, Ehret *et al.* 2001). The proper function of at least one of both factors is crucial for mammary gland development and lactation-dependent expression of milk genes (Hennighausen *et al.* 1997, Liu *et al.* 1997, 1998, Teglund *et al.* 1998, Groner 2002, Cui *et al.* 2004). Abolishing STAT5 factor-binding by point mutations in the GAS motif was found to abrogate the prolactin and dexamethasone-dependent induction of acetyl-coenzyme A-carboxylase- α (Acc- α) in the murine MEC line HC-11 (Mao *et al.* 2002).

STAT5 factors do not function alone. The lactation-specific expression of milk genes is under a complex regulation scheme, integrating not only a variety of

lactationally relevant hormones, like prolactin, hydrocortisone and insulin (Yu-Lee *et al.* 1998), but also signals from the extracellular matrix (Schmidhauser *et al.* 1990, Naylor *et al.* 2005). Hence, the promoters of milk genes are generally extended structures, containing clusters of binding sites for other transcription factors. These clusters are known as composite response elements (CoREs) and commonly embed a STAT5-binding site into attachment sites for NFI, C/EBP and palindromic half sequences of glucocorticoid response elements (Mukhopadhyay *et al.* 2001, Wyszomierski & Rosen 2001). All milk genes feature such CoREs in their proximal (~1 kbp) promoters. The immediate proximal core promoter of milk genes (~350 bp) contains often in addition attachment sites for other transcription factors, like Oct-1 (Groenen *et al.* 1992) and also YY1, which binds to the highly conserved 'milk box' (−140 to −110; Raught *et al.* 1994). Additional distal enhancers have been found in a variety of milk genes (Winklehner-Jennwein *et al.* 1998, Rosen *et al.* 1999), including also the promoter of the α S1-casein-expressing gene in rabbits, at around −3.5 kbp (Jolivet *et al.* 1996, Pantano *et al.* 2002). These distal CoREs may be highly relevant for the proper developmental control of milk gene expression, since inclusion of these distal CoREs was found to be mandatory, if casein promoters were used to drive mammary restricted and lactationally stimulated transgene expression in animals (Rijnkels *et al.* 1998, and discussion herein). Extending our previous characterization of this gene in cattle (Koczan *et al.* 1991), we have previously identified a CoRE in the far distant (~−10 kbp) α S1-casein promoter, featuring a doublet STAT factor-binding site with a repeat length of 21 bp, hence of exactly two turns of the DNA double helix (Herrmann 2001).

Binding of two STAT factor dimers in identical orientation in closest vicinity to each other provides a peculiar structural template for the attraction of auxiliary factors. It is known that STAT5 factors have to interact physically with coactivators to stimulate the expression of their target genes (Rosen *et al.* 1999). Regarding casein gene expression, they were shown *in vitro*, but not *in vivo* (Wintermantel *et al.* 2005), to cooperate not only with the glucocorticoid hormone receptor (GR; Stöcklin *et al.* 1996), mediated via C/EBP β (Wyszomierski & Rosen 2001), but also with the p300/CBP family of transcription-coactivators (Pfützner *et al.* 1998). Both GR and p300/CBP interact yet with another coactivator, NcoA-1 (Litterst *et al.* 2003). The p300/CBP factors and NcoA-1 share in common an intrinsic histone acetyl transferase (HAT) activity. Therefore, STAT5 factors stimulate gene expression in part by altering the chromatin structure in the vicinity of their binding sites.

Methylation of cytosine to 5methyl-cytosine in the context of CpG dinucleotides establishes a structural substrate for alterations of the chromatin structure. It imprints a tissue-specific pattern onto the DNA sequence and serves to properly silence genes according to the stage of development (Ng & Bird 1999). The methylated CpG bases are recognized by specific-binding proteins, like MeCP2, possessing a histone deacetylase activity (Nan *et al.* 1998). Deacetylation of histones causes tight condensation of the surrounding chromatin and consequently sustained silencing of transcription.

DNA methylation is known as an epigenetic regulator of gene expression, relevant for transcriptional silencing during development and genetic imprinting (Li 2002, Jaenisch & Bird 2003). Aberrant methylation plays a role in the development of chronic diseases and cancer (McCabe & Caudill 2005, Ushijima & Okochi-Takada 2005). Examples for *de novo* CpG methylation are mostly associated with the silencing of foreign DNA, introduced either by viruses or as artificial transgenes (Doerfler 2005). However, there are no examples demonstrating occurrence and regulatory significance of *de novo* methylation during an acute, physiological reprogramming of the cellular metabolism.

During the present study, we examined if alterations of the DNA methylation and of the chromatin structure around the CoRE in the remote α S1-casein promoter contribute to shut down casein synthesis after an acute experimental stimulus. We had seen in other studies examining host–pathogen interactions during mastitis, that a pathogenic strain of *E. coli*, causes acute mastitis within 24 h after application and abolishes casein synthesis in the infected quarter, but not in the uninfected control quarter of the same animals. Moreover, we observed in subclinical infections, that eventually single alveoli may be devoid of any α S1-casein mRNA, but at the same time express heavily immune defence genes (Yang *et al.* 2006), indicating a spatially restricted shutdown of casein synthesis.

Indeed, we prove that these STAT sites may bind STAT5 factors. We establish their functional potential in MEC models *in vitro*, and confirm their functional relevance *in vivo* on the basis of the lactation-specific alterations of CpG methylation, and chromatin compaction in their surrounding. We conclude that *de novo* methylation in MEC is very likely involved in the infection-induced shutdown of casein synthesis.

Materials and methods

Animals and udder samples

All animal experimentation was conducted in accord with accepted standards of humane animal care, and approved by the pertinent governmental authorities. Udder tissue was collected from pubertal ~16-month-old

heifers; from eight mid-lactating Holstein cows with experimentally induced mastitis and from two cows of an experimental herd, having been culled 10 and 14 days respectively after their first calving. These latter two cows had never been milked or suckled. Liver tissue was collected from the lactating cows. A full description of the mastitis model based on experimental infections of udders will be given subsequently (Petzl *et al.*, in preparation). Briefly, we infected eight healthy Holstein cows, all in the fourth month of their first lactation with low somatic cell counts (SCC) and absence of detectable bacterial growth. Intramammary challenge with 500 CFU of *E. coli* strain 1303 ($n=4$) or with 10 000 CFU of *S. aureus* strain 1027 ($n=4$) was performed after milking respectively in one-quarter 24, 12 and 6 h before culling. The bacterial strains had been previously isolated from cows with clinical mastitis. One-quarter was left as uninfected control. All animals infected with *E. coli* showed signs of severe mastitis 12 h after challenge like increased SCC, decreased milk yield, leukopenia, fever and udder swelling, while in the cows infected with *S. aureus* only slight clinical symptoms could be found. Blood samples were drawn from the jugular vein 24, 18, 12 h and immediately after challenge for serum preparation. Serum prolactin levels were determined using a bovine-specific RIA (Institute of Physiological Chemistry, Faculty of Veterinary Medicine, University of Leipzig, Germany). Udder tissue samples were collected from the cows under aseptical conditions between 5 and 10 min after culling. Washed udder skin was cut using an alcohol disinfected slaughter knife. A piece of tissue (5×5×5 cm) was taken out from a deeper area of the udder quarter followed by preparation of a 1×1×1 cm tissue piece from the centre of the probe under aseptical conditions. A small section (1×0.5×0.5 cm) hereof was preserved in RNA later (Qiagen) for RNA analysis. Two adjacent sections were either snap-frozen in liquid N₂ for DNA and protein analysis, or fixed in paraformaldehyde for histological examination.

Retrieval and sequencing of the distal α S1-casein promoter

The distal α S1-casein promoter was retrieved from the λ phage λ bzslCNII, which had been isolated in the course of the initial description of the bovine α S1-casein-encoding gene (Koczan *et al.* 1991, Koczan 1994). The new sequence was established from subclone 300 (in pUC18), comprising the entire promoter on its 11 kbp genomic insert. Further, subclones and DNA sequences were established with conventional methods, using pKS+ (Stratagene, La Jolla, CA, USA) as vector and ABI-sequencing chemicals and sequencer (ABI310; Applied Biosystems, Foster City, CA, USA).

Cloning of α S1-promoter containing reporter genes

The firefly luciferase expressing reporter gene harbouring the proximal promoter had been established in previous experiments (Koczan 1994). It contains 1608 bp of the promoter, from the genomic SphI site at position –1608 to the BglII site +89 in the 5'-UTR of the α S1-encoding gene. This segment was recloned into pGL3-basic (Promega). The distal promoter segment (from positions –10 507 to –9696) was amplified with PCR techniques from subclone 305 of the original λ -isolate, using the vector-binding T7-oligonucleotide primer in the forward direction, and the oligonucleotide 5'GCCCGGTACCAGGAAAGGAGTCCTGAAAGATG, introducing a KpnI restriction site (underlined). After subcloning into pGEM-Teasy (Promega), the insert was retrieved as KpnI fragment, cloned 5' to the proximal promoter segment in CasA, exploiting the KpnI site of the vector. Proper orientation of the insert was verified via sequencing.

Southern analysis

Restriction digestion and southern hybridizations followed conventional techniques. Ten micrograms of restriction-digested genomic DNA were loaded into each lane. Hybond N (Amersham) was used as blotting membrane. The radioactively (³²P-dCTP) labelled hybridization probe was the same distal promoter segment, as cloned into the expression construct. Stringency of the post-hybridization washes was defined by 0.1× SSC and 65 °C (2×30 min). Radioactive signals on the filters were detected with the Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts, probe labelling, super shifts and the general procedures for the EMSA analysis were conducted as described (Mao *et al.* 2002). To generate the double-stranded probe, we used an oligonucleotide conforming to the sequence of the doublet STAT-binding site, as given in Fig. 1A, annealed to it the oligonucleotide 5'-TTGAACCTTGA and filled in ³²P-dCTP with the Klenow enzyme. STAT5A was super-shifted with 1 µg per assay of the antibody sc-1081 (Santa Cruz, CA, USA).

Cell culture, transfection, hormone induction and reporter gene assays

The establishment of pools of clones harbouring the stably transfected reporter gene constructs in the established murine MEC line HC-11 was essentially as described (Mao *et al.* 2002). Briefly, cotransfection of

Figure 1 Genomic map, structure and developmental alterations of CpG methylation around the distal α S1-casein enhancer. (A) Distribution of STAT-binding sites (lines with caret) in the α S1-casein promoter, all CpG dinucleotides (knobbed lines) and promoter segments (stippled and grey boxes) fused to establish the reporter gene constructs (upper); sequence of the tandem STAT-binding sites (middle, STAT-core motif underlined and bold face letters; putative-binding sites for HMGB1, broken line); detail of the area analysed (lower). Indicated are the positions of the PstI and HpaII restriction sites used for the southern analysis. Binding sites for STAT factors (lines with caret), GR and C/EBP (knobbed and double-knobbed lines respectively) are also indicated. The bracket above the sequence denotes the DdeI restriction enzyme-binding site. Arrows denote the position of CpG dinucleotides. Boxed areas are segments analysed by the bisulphite technique (hatched) and used as probe for the southern analysis (grey). (B) Southern analysis of mammary gland extracted DNA, digested with PstI (P) alone or combined with MspI (Ms) or HpaII (Hp). MspI and HpaII both cut the DNA sequence CCGG, but HpaII cutting is blocked by C-methylation. The DNA was collected from pubertal (pub), fully lactating (lact) or a cow, 10 days after having given birth to a calf but was never suckled or milked (n. lact). Liver tissue was from the lactating cow. The expected sizes of the PstI and the longer product of the PstI/HpaII double digests are indicated. (C) Quantitative determination of the percentage of C-methylation with the bisulphite technique, applied to the same set of DNA samples, as used for the southern analysis. The values measured for each of the three different CG dinucleotides contained in this area are represented by different textures of the columns. The sequence of the elongated α S1-casein promoter has been submitted to the EMBL database under accession number AJ812028 and used to update file X59856.

the respective experimental reporter gene constructs with pSV2neo (Clontech) was used to select for G418-resistant clones. Pools (>500) were grown to confluency in a medium without insulin and kept in this medium for 2 days. EGF was washed out and cells were cultured for 2 days in EGF-free medium containing 5% FCS and insulin, with, or without, 5 μ g/ml ovine prolactin (Sigma) and 0.1 μ M dexamethasone (Sigma; cf. Lee *et al.* 1998, Winklehner-Jennwein *et al.* 1998). Firefly-luciferase activity was measured from cell extracts using the dual-luciferase reporter assay system (Promega) and a Berthold luminometer, as prescribed. Protein concentrations of the extracts were determined with the Bradford system (Bio-Rad).

Bisulphite modification of genomic DNA and direct PCR sequencing

The percentage of 5methyl-cytosine in DNA was determined with the bisulphite technique. Bisulphite can convert cytosine (C) into uracil (U) under conditions where methylated cytosines are unreactive. PCR amplification will eventually replace uracil with thymine (T). The bisulphite sequencing protocol was essentially as described (Vanselow *et al.* 2005). The modified target sequences were amplified by PCR with primers that specifically bind to the plus strand of the corresponding genomic regions. Amplification results in the generation of a mixture of molecules harbouring either C or T bases at CpG dinucleotides. This causes the occurrence of C/T polymorphisms at these positions in subsequent sequencing reactions. Primers used to amplify 269 bp of the distal promoter of α S1-encoding gene (positions -10 434 to -10 165) were 5'-TTGGTTAGGGAATTGGATTATTATGTT (forward) and 5'-ATTCCAACAATTCACAACATATATTCCT (reverse). Primers used to amplify 453 bp from promoter 2 of the bovine *Cyp19* gene (used as a control, cf. Vanselow *et al.* 2005) were 5'-TGGA-GAAGTTTATTTTTTGGGGGAAGT (forward) and 5'-TCCCCAAAATATACA-TTTAAACTCCT (reverse). PCR was performed in 25 μ l reaction volume with 0.25 U Taq polymerase (Qbiogene, Heidelberg, Germany). The first cycle was 94 $^{\circ}$ C for 5 min, 53 $^{\circ}$ C for 30 s, 70 $^{\circ}$ C for 2 min. This was followed by 35–40 cycles of 94 $^{\circ}$ C for 30 s, 53 $^{\circ}$ C for 30 s, 70 $^{\circ}$ C for 2 min. The last PCR step was 70 $^{\circ}$ C for 5 min. Prior to sequencing PCR fragments were pre-analysed on a 4% agarose gel stained with ethidium bromide and quantified (GeneQuant II Instrument, Pharmacia).

Direct sequencing of PCR products was performed with a LI-COR 4200 Series DNA Analysis System (MWG, Heidelberg, Germany) using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia). The IRD800-labelled (MWG BIOTECH, Ebersberg, Germany) sequencing primers

for the PCR fragments were 5'-AAGTGTTATAAGTAA-GATTTGGTATAGT and TAAAGTAAGGTAGTTG-GGGATTGTG for α S1-casein and Cyp19 respectively.

The sequence gel files were further processed and evaluated with the PHRED software, version 0.020425 (53;54) in order to quantify the C/T polymorphic sites (Vanselow *et al.* 2005). The percentage of methylation was calculated as the portion of the C peak area within each polymorphic CpG site [% methylation = (peak area of C/peak area of C + peak area of T) $\times$ 100].

For comparative statistical analysis (*t*-test), the SigmaStat software (SPSS Science Software GmbH, 40699 Erkrath, Germany) was used.

CHART-PCR

Chromatin compaction was measured with the CHART-PCR technique (Rao *et al.* 2001). Under liquid nitrogen, 50–100 mg samples were powdered and resuspended in 3 ml ice-cold resuspension buffer (RSB) (10 mM Tris (pH 8.0), 3 mM MgCl₂ and 10 mM NaCl), containing 0.5% NP40 and a proteinase inhibitor cocktail (Roche) with phenyl-methyl-sulphonyl-fluoride (PMSF) (1 mM). After incubating for 5 min on ice, the tissue was homogenized with a Dounce homogenizer. Sucrose was added to 0.2 M and the chromatin was spun down in an Eppendorf centrifuge (10 min, 1100 $\times g$, 4 °C). The pellet was washed once in RSB buffer, containing 1 mM mercaptoethanol and resuspended in 200 μ l recommended buffer for restriction digestion with DdeI (Promega). A small aliquot ($\sim$ 10 μ l) was removed to determine the DNA concentration. A volume containing 2 μ g DNA was removed, filled up with restriction enzyme buffer to 150 μ l. Twenty units of DdeI were added and the assays incubated for 30 min at 37 °C. A control sample was prepared and treated similarly, but no enzyme was added. Subsequently, the DNA was purified after digesting the mixture over night (56 °C) with proteinase K (40 μ g per assay; Roche), after addition of a similar volume of double concentrated Proteinase K digestion buffer (fc: 50 mM Tris (pH 8); 0.15 M EDTA; 0.1 M NaCl; 6 mM DTT, 1% SDS). DNA was extracted and purified with phenol and finally dissolved in 50 μ l water. The DNA concentration was measured with a Nanodrop spectrophotometer.

The quantity of undigested target DNA was measured with the LightCycler real-time PCR instrument (Roche), essentially as described (Goldammer *et al.* 2004). The primers (forward 5'-AACAAATCCATGAC-CATCCTGAC; reverse AGGAAAGGAGTCCTGAAAGATG) amplified a 386 bp comprising segment of the α S1-casein with the STAT5-binding area of the α S1-casein promoter in its centre. Relative copy numbers were calculated according to a dilution series of a reference subclone of the respective promoter area.

Values are expressed as percentile fraction of the DdeI digested versus undigested control samples.

Real time PCR quantification of the α S1-casein and β -defensin mRNA

The abundance of α S1-casein mRNA molecules was measured from TRIZOL (Invitrogen) extracted RNA samples, also using the LightCycler instrument (Roche). The oligonucleotide primers 5'-CTTTTCA-GACAATTCTACCAGCT (forward) and 5'-CAT-AACTGTGGAGTCCCTCAG amplified 191 bp from a cDNA aliquot equivalent to an input of 75 ng total RNA, per assay.

The abundance of β -defensin mRNA copies was assessed with the primers 5'-AGGCTC CAT-CACCTGCTCCT (forward) and 5'-GAAACAGGTGC-CAATCTGTCTC (reverse), amplifying 159 bp from cDNA copies derived from the BNBD4, BNBD5, EBD- and LAP-encoding genes. The use of these conserved primers provides a robust measure for the expression level of members from the β -defensin-encoding gene family. LAP-derived copies contribute 80% of all amplicates in the infected udder, as revealed by subcloning and subsequent sequence analysis (WY, unpublished observation).

Two-dimensional gel electrophoresis (2D-GE)

Two-dimensional gel electrophoresis was performed as described (Gorg *et al.* 2004). Samples were lysed in 100 μ l lysis buffer (9 M urea; 4% CHAPS; 1% DTT; 1 mM EDTA; 0.8% immobilized pH gradient (IPG)-buffer, Amersham; complete protease inhibitor cocktail, Roche) and the cells were disrupted using the 2D sample grinding kit (Amersham) according to the manufacturer's instruction. Rehydration buffer was added (240 μ l of 8 M urea; 2% CHAPS; 0.5% IPG buffer, Amersham; 0.28% DTT) samples soaked into isoelectro focusing (IEF) gel strips on a IPGphor apparatus (Amersham Biosciences) for 12 h at 50 V. Electrophoresis was performed at 50 μ A/gel strip, according to the following procedure: 500 V for 1 h, 1000 V for 1 h and 8000 V for 4 h.

After the isoelectric focussing, the IPG strips were equilibrated with gentle agitation in a buffer containing 1.5 M Tris (pH 8.8); 30% glycerol; 6 M urea; 2% SDS, 1% DTT, followed by a second incubation in the same buffer, except that DTT was substituted by iodoacetamide (2.5%, 15 min for each buffer). The second dimension run was performed on 12.5% acrylamide gels according to Laemmli (1970). Gels were stained with Coomassie brilliant blue R250.

Analysis of 2D gels

The positions and protein quantities of spots resolved on the 2D gels were analysed with the 2D gel analysis software (DECODON, Greifswald, Germany). For each gel, the spots were detected and quantified automatically. In all cases, an additional manual spot detection was performed to verify the automatically obtained results. Each spot was sampled from four independently generated gels. The protein quantity of the individual spot was calculated relative to the sum of all detectable protein spots per gel set as 100%.

Mass spectrometry

The identity of casein subtypes was verified with mass spectrometry.

In gel digest of proteins

Excised gel spots (1.75 mm in diameter) were placed into 96-well microtitre plates and digested with trypsin. Tryptic digests and subsequent spotting on a matrix-assisted laser desorption ionization (MALDI) target was carried out automatically with the Ettan Spot Handling Workstation (Amersham Biosciences), using the following protocol. The gel pieces were washed twice with 100 µl solution of 50% CH₃CN and 50% 50 mM NH₄HCO₃ for 30 min and once with 100 µl 75% CH₃CN for 10 min. After drying at 37 °C for 17 min, 10 µl solution containing 20 ng/µl trypsin (Promega) was added and the mixture incubated at 37 °C for 2 h. For extraction, gel pieces were covered with 60 µl 0.1% TFA (trifluoro acetic acid) in 50% CH₃CN and incubated for 30 min at 40 °C. The peptide containing supernatant was transferred into a new microtitre plate and the extraction was repeated with 40 µl of the same solution. The supernatants were completely dried at 40 °C for 220 min. The dry residue was dissolved in 3 µl of 0.5% TFA in 50% CH₃CN and 0.4 µl of this solution was directly spotted on the MALDI target. Next, 0.4 µl of a saturated α -cyano-4-hydroxy cinnamic acid solution in 70% CH₃CN was added and mixed with the sample. The samples were allowed to dry on the target 10–15 min before the measurement.

MALDI-TOF-MS

The molecular masses of tryptic digests were measured by matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS), using the Proteome-Analyser 4700 (Applied Biosystems, Foster City, CA, USA). Peak lists were created with the peak to mascot script of the 4700 Explorer Software

The peptide masses were used to search the databases (Swiss-Prot or NCBI) to identify proteins, using the search program MASCOT (www.matrixscience.com).

Western analyses

The primary antibody was raised in rabbits against bovine α S1-casein, purified from cow milk using preparative gel electrophoresis. The principal techniques for antigen preparation and immunization have previously been described (Seyfert & Sawatzki 1986). In order to detect α S1-casein, udder samples (10 µg) were resolved on conventional 12.5% Laemmli gels (Laemmli 1970) and blotted onto PVDF membranes (Millipore, Germany) using a semi-dry blotting apparatus with a three-buffer system (anode buffer 1: 0.3 M Tris-HCl (pH 10.4), 10% methanol; anode buffer 2: 0.25 M Tris-HCl (pH 10.4), 10% methanol; cathode buffer: 25 mM Tris-HCl (pH 9.4); 40 mM 6-amino-*n*-caproic acid, 10% methanol) for 1 h at 1 mA/cm² of the membrane. Blots were handled as described (Tomek *et al.* 2002). They were incubated with 100 ng/ml affinity column purified first antibody, 50 ng/ml secondary HRP-labelled antibody against rabbit IgG (Cell Signalling Technologies, Frankfurt/Main, Germany) and developed with the ECL detection reagent according to the manufacturer's instruction (GE, Freiburg, Germany).

Results

Structure of the distal composite response element (CoRE)

Eight consensus STAT factor-binding sites are located on 10 kbp of the α S1-casein promoter. Their distribution, the DNA sequence of the doublet STAT-binding site and the arrangement of CpG dinucleotides is shown in Fig. 1A. Central feature of the distal composite response element is the peculiar tandem arrangement of two STAT-binding sites. They are separated by 21 bp, i.e. exactly two turns of the double helix. This unusual configuration warranted a more detailed examination of this area. The computer analysis of the sequence (Quandt *et al.* 1995) reveals two potential binding sites for glucocorticoid receptor C2C2 zinc finger proteins and a C/EBP attachment site. Hence, this DNA sequence area features most components known to constitute a CoRE. It may be noteworthy, that the computer analysis identifies also an attachment site for the high-mobility group I factors, overlapping with tandem STAT-binding sites. These factors bind to nucleosomes and eventually unfold the chromatin in their vicinity (Bustin 2001).

The DNA sequence motif of this CoRE is quite unique. Blast searches against all available mammalian data bases using either 70 bp with the sequence as shown in Fig. 1A in the centre, or comprising this element on a longer search sequence (1195 bp; from position -10401 to -9212) devoid of retrotransposons does not reveal any significant homologies. The analysis reveals individual STAT-binding sites on short sequence elements (<20 bp), if the short search is used, but does not reveal any doublet STAT-binding sequence motif. The short and long search sequences both line up with the 5'-flanking region of the human α S1-casein-encoding gene, at around -13 000. However, while the observed 64.2% sequence similarity over 1195 bp may indicate some evolutionary conservation of this area, this human sequence does not feature any STAT-binding motifs (defined as TTCNNNGAA) in the aligned area.

DNA-methylation in the vicinity of the STAT factor-binding site is developmentally regulated

We used the methylation sensitive restriction endonuclease HpaII to survey the status of DNA methylation in the vicinity of the STAT-binding sites. The southern blot reveals (Fig. 1B), that most of the DNA is uncut in DNA from liver, hence methylated at the HpaII site. The degree of methylation is less in mammary glands of pubertal cows. The udder is fully built up during pregnancy and develops to a gland ready for lactation and casein synthesis, but not lactating yet. The HpaII site is almost completely demethylated in the fully lactating udder. In contrast, the DNA retrieved from udder of a cow, which had given birth to calves, but had never been milked reveals a degree of methylation similar to the pubertal, but not lactating udder. The same result was obtained in a repetition of experiment with a comparable set of different animals.

We quantified the percentage of methylated DNA molecules in the crucial area with the bisulphite technique from those samples used for the southern analysis. We choose to analyse a short segment 5'-adjacent to the doublet STAT site, an area comprising three CpG dinucleotides. All the three are methylated to approximately the same extent in any given tissue (Fig. 1C). The mean values confirm quantitatively the qualitative images, as obtained from the southern analysis. More than two-third ($66.7 \pm 4.0\%$) of the DNA molecules from the liver tissue are methylated, but only $10.2 \pm 0.5\%$ are methylated in the fully lactating gland. The values for the udders from the pubertal and not lactating glands are $42.2 \pm 2.8\%$ and $36.3 \pm 1.5\%$ respectively. These data show that lactation coincides with hypo-methylation of the CoRE in the α S1-casein promoter.

The remote STAT sites bind STAT5A and enhance lactational stimulation of α S1-casein promoter constructs in HC-11 cells

We used the EMSA technique to identify the nature of STAT factors binding to the doublet STAT site. Expectedly, nuclear extracts from lactating udders shift the radioactively labelled probe harbouring this sequence motif (Fig. 2A). The complex is completely supershifted by a STAT5A-specific antibody. We have previously shown with detailed mutational analyses of STAT-DNA sequence motifs that the antibody used is indeed highly specific for the bovine STAT5A factor

Figure 2 The enhancer binds STAT5A and increases α S1-casein promoter inducibility by lactational hormones. (A) EMSA analysis with nuclear extracts from lactating udders. The radioactively labelled probe (sequence as given in Fig. 1A, middle panel) was loaded, without addition of proteins (FP) or after mixing with nuclear extracts (lane S, arrow). The shifted complex is entirely supershifted by the addition of a STAT5A-specific antibody (lane SS, knobbed arrow) or competed away by a 50-fold molar excess of the unlabelled probe (lane C). (B) The distal enhancer increases the inducibility of a 1.6 kbp proximal segment from the α S1-casein promoter by lactation hormones. Luciferase expressing reporter genes with (black columns) or without (open columns) 811 bp of the distal enhancer were stably transfected into the murine MEC model cells HC-11. Pools (>500 clones for each construct) of stably transfected clones were grown to confluency and subsequently stimulated for 48 h with dexamethasone (dex), or prolactin (prl), or a mixture of both, as indicated below. The luciferase activity of the extracts was normalized according to the protein content of the sample. Represented are the mean values (error bars, S.E.M.) from three different independent experiments. Asterisks, significance levels of difference to unstimulated control (* $P < 0.05$; † $P < 0.01$; t -test).

(Mao *et al.* 2002). Hence, this DNA sequence element can bind activated STAT5A complexes, as retrieved from lactating udders.

We examined next the functional contribution of the doublet STAT5-binding site to transduce signals from lactation hormones onto the α S1-casein promoter. We compared the potential of the glucocorticoid analogue dexamethasone and prolactin, alone and in combination, to induce the expression of two different α S1-casein reporter genes, which had stably been transfected in to HC-11 cells. The reference construct comprised a 1.6 kbp segment of the proximal promoter. This promoter segment harbours the most proximal STAT5-binding site at around position -100. For comparison, we had fused to this construct the distal CoRE on a 811 bp comprising segment including the doublet STAT5-binding site (cf. Fig. 1A). The elongation of the proximal promoter does not alter the basal level of expression, if pools of stably transfected cells are compared (Fig. 2B). However, both constructs differed in the extent of their inducibility by the lactationally relevant hormones. While prolactin alone stimulates the expression of neither construct (induction folds equal 1.0 for both), the elongated construct with the additional STAT5 doublet site is much stronger induced by the combined action of prolactin and dexamethasone than the short construct (5.0-fold vs 1.9-fold, long versus short construct respectively). The stimulating effect of dexamethasone alone is also somewhat more pronounced for the larger construct (2.2-fold vs 1.6-fold induction respectively). The data together prove in model cells the functional potential of this remote enhancer element. This adds physiological significance to the observed lower methylation levels of the surrounding DNA sequence in lactating udders. It suggests that this process regulates the accessibility of the DNA-binding sites for the receptors of those hormones, known to be highly relevant for induced α S1-casein expression during lactation.

Acute mastitis blocks casein synthesis and reduces α S1-casein mRNA abundance

We chose to analyse a possible contribution of DNA methylation of this distal enhancer to block casein synthesis in samples from udder quarters of fully lactating cows, which had experimentally been infected with *E. coli* pathogens. We focused the analysis on this particular CoRE, since we had already seen that the methylation status of this area is developmentally regulated in concert with lactation. Moreover, the doublet STAT5-binding sites are surrounded by several CpG dinucleotides (cf. Fig. 1A), discriminating this area of the promoter from the vicinity of the proximal STAT5-binding site (at -95), where the nearest CpG

dinucleotide is more than 500 bp upstream from the STAT5-binding site. The remote location of this CoRE at -10 kbp suggests in addition that transcription factors binding here might be related to prolactin signalling rather than contributing to basic promoter functions, in contrast to the proximal STAT5-binding site. The experimental set-up included to infect sequentially, in any cow the udder quarters with 500 CFU of the pathogen at times 0, 12 and 18 h and leaving one quarter as uninfected control. The udders were sampled 24 h after the first infection. The pathogen strain used for these infections elicits acute mastitis within 24 h after infection of the udder quarter. This

Figure 3 Qualitative and quantitative determination of casein synthesis and α S1-casein mRNA during infection with *E. coli*. (A) Two-dimensional resolution of proteins extracted from different udder quarters of the same animal. The quarters had either been not infected (control) or harboured the pathogens for 12 or 24 h. Labelled are large protein spots from BSA, α S1-, or β -casein and β -lactoglobulin (β -LG), as verified by MS-MALDI-TOF spectrometry. (B) Western blot of all udder samples developed with a α S1-casein-specific antibody. Samples (10 μ g) from the different udder quarters, as indicated were resolved on 10% Laemmli gels, blotted and α S1-casein detected with the specific antibody. Asterisks denote samples also shown above. (C) Mean values (n , 4; error bars, S.E.M.) of the relative abundance of α S1-casein (carets) and β -casein (squares), as determined from 2D gels, relative to the means from control quarters, set as 1. (D) Decay of α S1-casein mRNA abundance, as determined by real time PCR. Represented are the mean values (n , 4; error bars, S.E.M.) of α S1-casein mRNA molecules at anyone time, as measured per weight equivalent of cDNA input. The 24 h value differs from all others with high significance ($P < 0.05$ – 0.008 ; t -test).

causes very consistently a complete shutdown of casein synthesis in the infected quarter, but not in the uninfected control quarters. The two-dimensional gel electrophoresis shows large spots of caseins in the uninfected control quarter, as well as in the quarter infected for only 12 h, while the caseins are almost entirely absent in that quarter of the same animal, which had been infected 24 h before (Fig. 3A).

Western blots probed with an antibody raised against bovine α S1-casein from milk confirmed qualitatively a steep decrease of the α S1-casein content in the samples taken 24 h after infection (Fig. 3B). While the α S1-casein signals from the 0 to 12 h samples are all of about the same intensity, they are much lower in the 24 h samples, barely detectable in three from among the four samples.

Quantification of the casein spots resolved in two-dimensional electrophoresis from all of the four animals showed that the average casein content remains constant during the first 12 h post-infection. However, then it drops sharply down to less than 10% of the uninfected control (Fig. 3C). Decreased casein concentration in these samples indicates reduced synthesis, since these proteins are secreted and the udders had been milked out before sampling. The α S1-casein mRNA abundance is also decreased by the infection, but only to 53% in the quarters infected for 24 h (Fig. 3D). Analysis of all samples reveals an almost linear decrease of the α S1-casein mRNA with the time of infection, from $143 \pm 24 \times 10^6$ copies down to $76.6 \pm 5 \times 10^6$ copies. These data together show that infection causes an abrupt cessation of casein synthesis initiated after a lag period of ~ 12 h. Interestingly, they reveal a much steeper decrease of the casein protein concentration than mRNA abundance. This disproportion of both time course and extent between the decrease of α S1-mRNA abundance and protein synthesis indicates the operation of a strong translational control mechanism to shut down α S1-casein synthesis in addition to reduced transcription.

The histological examination of the sections taken from the udder tissue 24 h after infection shows alterations known to be associated with acute mastitis (Fig. 4). These include oedema of septa separating bundles of alveoli, disintegration of alveoli and filling of alveoli with granulocytes. Importantly, however, they reveal also that the spongy architecture of the secreting milk parenchyma is principally well preserved, still featuring MEC as prevailing cell type.

The infections did not decrease the systemic levels of circulating prolactin. These values were determined from blood serum sampled at various times during the experiment. The prolactin levels were 5.6 ± 2.04 ; 8.0 ± 2.71 ; 5.3 ± 2.15 and 6.1 ± 0.96 ng/ml of serum (mean + S.E.M.), sampled at times 0, 12, 18 and 24 h after the first infection.

Figure 4 Comparison of the udder tissue structure in control and *E. coli* infected quarters. Representative low magnification ($100\times$ primary magnification) images from haematoxylin/eosin-stained specimen taken from quarters of the same animal, either from the uninfected control (A, B), or after 24 h infection (C, D). Clumps of granulocytes recruited into alveoli and oedema of septa are obvious in the infected quarter (C, arrow and asterisk respectively), as well as disintegrating alveoli (D, arrow).

The DNA surrounding the remote STAT5 sites is remethylated to the degree of not lactating udders within 24 h after infection, resulting in chromatin compaction

We measured the degree of CpG methylation in the samples from the uninfected control udder quarters, and from those infected for 12 and 24 h respectively. The degree of methylation found in the control quarters ($n=4$, $10.5 \pm 1.4\%$, Fig. 5A) was as low as previously observed from healthy glands at full lactation (cf. Fig. 1C). However, methylation was increased to $47.4 \pm 2.4\%$ in the samples from the quarters harbouring the pathogens for 24 h, hence to very similar levels as recorded from the pubertal or not lactating glands from the previous experiments. The quarters having been infected for 12 h only reveal an intermediate degree of methylation ($18.4 \pm 5.1\%$). The individual variation is large in these samples and only two of the four animals displayed at 12 h already a significantly increased proportion of methylated DNA molecules.

Increased CpG methylation is assumed to result in chromatin condensation. Hence, we used digestion of chromatin with limited amounts of the restriction enzyme DdeI to compare in CHART-PCR the degree of chromatin condensation around the STAT5 CoRE in the samples from the control quarters to those taken 24 h after infection. The chromatin from the control quarters was much less protected against digestion than that of the samples from 24 h infected samples ($36.7 \pm 1.4\%$ vs $73.9 \pm 10.1\%$, mean values of control versus 24 h samples respectively). Moreover, the degree of methylation correlates significantly (r , 0.81; P , 0.017)

Figure 5 Alterations of CpG methylation and chromatin condensation in the vicinity of the α S1-casein enhancer. (A) Mean values (error bars, S.E.M.) of the three CpG motives, represented for each of four animals (individuals are specified by the texture of columns), as measured from udder quarters having harboured the *E. coli* pathogen for the time, as indicated. Numbers below the columns represent the number of somatic cells per ml of milk, as collected from each quarter just prior to sampling the udder tissue for DNA preparation. (B) The degree of chromatin compaction correlates with the degree of methylation. The degree of methylation (abscissa) of control (triangles) or 24 h infected samples (squares) is plotted against the degree of chromatin protection against DdeI restriction (ordinate, from CHART-PCR). Mean values from duplicate CHART-assays of anyone sample are indicated. The correlation is obvious (r , 0.81) and statistically significant (Pearson product moment correlation; P , 0.017).

with the degree of chromatin protection, as measured in the CHART-PCR (Fig. 5B). Thus, the infection-related increased degree of methylation resulted in chromatin condensation around the CoRE.

To examine, if the infection-related alterations in the percentage of methylated DNA molecules are specific for the α S1-casein, we measured in these udder samples the degree of methylation at three CpG sites on the promoter 2 of the *Cyp19* aromatase-encoding gene (Vanselow *et al.* 2005). The *Cyp19* aromatase gene is not known to be expressed in udder or to be lactationally regulated. We found all these sites highly methylated, to the same degree in all samples (85.6 ± 4.9%, 85.9 ± 2.6%, 87.4 ± 4.2%, control, 12 and 24 h samples respectively). Moreover, we measured meanwhile with CHART-PCR from the same samples, as used in this study, that the degree of chromatin compaction the promoters from the BNBD5- and LAP-encoding genes

is regulated just conversely to the α S1-casein promoter. The β -defensin promoters are highly compacted in the uninfected control quarters, where these genes are not expressed, while their chromatin is less compacted in the 24 h infected quarters, where also these genes are highly expressed (Liu, S; thesis at the FBN; unpublished observation).

These observations together show, that the modulations in the degree of methylation and chromatin compaction around the CoRE are specific for the α S1-casein promoter and not caused by generalized, genome-wide alterations of the degree of DNA-methylation at any promoter in these samples.

The percentage of α S1-casein promoter methylation is independent of the amount of granulocytes recruited into the udder

Infection of the udder causes a substantial influx of immune cells into the udder, mainly polymorphonuclear granulocytes (PMN; Paape *et al.* 2000). These cells are not synthesizing α S1-casein and thus conceivably harbour this promoter in its highly methylated form. Hence, increasing proportions of these cells in whole udder extracts might increase the percentage of DNA molecules with a methylated α S1-casein promoter. Two experimental lines of evidence show that the amount of PMNs inside the udder quarter does not significantly influence the percentage of methylated promoter molecules.

First, we had recorded the number of somatic cells in the milk of the individual udder quarters at the time of sampling (Fig. 5A). In neither of the three time groups (control, 12 and 24 h infected) is there any indication that the proportion of methylated α S1-promoter molecules might correlate positively with the number of milk cells. We observed in the 24 h infected samples, for instance, that the animal with highest percentage of methylated DNA molecules contained only 1×10^6 cell/ml of milk, while 5×10^6 of cells per ml of milk were found in the animal featuring the least degree of α S1-casein promoter methylation.

Second, we analysed samples from animals after experimental infections of udder quarters with a pathogenic *S. aureus* strain. This strain causes subclinical rather than acute mastitis and decreases milk and casein synthesis only by ~30%, even after prolonged (e.g. 84 h) periods post-infection. However, large numbers of granulocytes are eventually recruited into the infected quarters. We determined the degree of CpG methylation around the remote STAT-binding sites of the α S1-casein promoter in three udder quarters from the same animal, which differed regarding infection status and milk cell numbers. One-quarter was the uninfected control; in a second one the infection was not manifested while the third quarter featured a subclinical mastitis, 84 h after

Table 1 Degree of CoRE methylation and accompanying parameters from an udder infected with *S. aureus*

	% Methylation	Somatic cells (10^3 /ml)	α S1-mRNA copies (10^6)	β -Defensin mRNA copies
Udder quarter				
Front left	10.2	34	367	1708
Front right	18.5	164	374	1076
Back left	18.4	1592	389	15005

infection. This quarter, but not both others, was clearly reprogrammed to immune defence, as indicated by a 15-fold increased expression of bactericidal β -defensin-encoding genes over the basal level found in the other two quarters (Table 1). The numbers of cells in the milk collected immediately before sampling the udder tissues varied between 0.04 and $1 \times [10^6]$ cells per ml of milk respectively. However, the degree of methylation at the α S1-casein promoter was low in all quarters and similar to all our other samples from fully lactating udders (Table 1). Likewise, similar and high were the abundances of the α S1-casein mRNA molecules in the three samples.

The data together show that the degree of methylation of the α S1-casein promoter does not correlate with the amount of milk cells in these samples, nor is it influenced by the event of infection itself. Rather, it correlates with decreased casein synthesis and α S1-mRNA abundance. Considering the fact that MEC are still a prevailing cell type within the first 24 h after infection, our data indicate clearly that the α S1-casein promoter must be remethylated in MEC by *de novo* methylation of CpG-dinucleotides in association with the shutdown of casein synthesis.

Discussion

We describe a novel enhancer in the far upstream promoter of the bovine α S1-encoding gene, prove its functional significance and find, as key result, that CpG remethylation in its vicinity is specifically associated with an udder quarter restricted shutdown of α S1-casein synthesis.

The detection of a distal enhancer in the far upstream region of the bovine α S1-casein promoter adds another example of such a *cis*-relevant element. However, an unusual feature is the very special arrangement of two STAT5-binding sites in tandem, spaced apart by only two turns of the DNA helix. This structure may bind two STAT5 dimers in similar orientation. Hence, it provides not only a firm template for STAT5 factor multimerization, known to enhance STAT factor action (Vinkemeier *et al.* 1996, John *et al.* 1999), but also ensures their proper orientation for effective interaction with additional cofactors.

The far upstream enhancer is functionally significant *in vivo*

It is not compelling to assume *a priori* that a regulatory element identified 10 kbp upstream of a promoter confers a significant function *in vivo*. A first suggestion for a biological function of this area stems from our *in vitro* experiments comparing the hormonal induction capacity of the α S1-casein promoter with or without the distal enhancer. The fact that only the combined administration of prolactin and the glucocorticoid hormone analogue dexamethasone resulted in an improved inducibility of the enhancer carrying construct is not surprising, since similar results had previously been obtained with comparable constructs in HC-11 model cells (Doppler *et al.* 1989, Schmitt-Ney *et al.* 1991, Lechner *et al.* 1997). The observed enhanced hormone responsiveness conferred by the distal CoRE agrees with our earlier results including the entire > 10 kbp promoter sequence, where we had found that bovine α S1-promoter constructs were only inducible by the combined action of prolactin and dexamethasone in stably transfected HC-11 cells, if they comprised 5 kbp or more of 5'-flanking sequence (Koczan 1994). The distal CoRE at -10 kbp quite likely specifies one control element highly relevant to this observation.

However, compelling evidence for the biological significance of this far upstream enhancer *in vivo* is provided by our observation that the methylation status of this area in the α S1-casein promoter is developmentally regulated in a lactation-specific fashion. This epigenetic mechanism obviously contributes to establish the competence of the promoter for lactation-specific regulation. Indeed, it has previously been postulated that mechanisms other than prolactin-activated STAT5 signalling must contribute to high-level expression of milk genes in the mammary gland of the cow (Wheeler *et al.* 2001). These authors had found that in cattle, but not in the mouse, neither the abundance of STAT5 factors and their mRNAs, nor the binding activity of these factors retrieved from the udders correlate closely with the extent of lactation. This was found in analyses of both the developmentally governed lactation cycle and the lactating individuals (Wheeler *et al.* 1997, 2001).

A very short promoter segment (105 bp) of the rat β -casein promoter, devoid of any distal CoRE, was found to be stimulated by prolactin in the COS7

reconstitution system of STAT5-mediated prolactin signalling (Gouilleux *et al.* 1994). However, assigning a clear biological significance to a far distal CoRE in the α S1-casein promoter is in keeping with previous experiences gained, when casein promoters were used to drive mammary gland-specific expression of transgenes. It was found that short rat β -casein promoter constructs (<3 kbp) express the transgene only at a low level in the mammary gland (Lee *et al.* 1988, Bühler *et al.* 1990), while high level and lactationally stimulated expression was observed using 3.8 kbp (but not 1.7 kbp) of the bovine β -casein promoter (Cerdan *et al.* 1998) or ~21 kbp of the bovine α S1-casein promoter (Meade *et al.* 1990). The necessity to use extended casein promoter segments for strong, mammary gland and lactation-restricted expression of transgenes has been confirmed ever since (Rijnkels *et al.* 1995, 1998, Brophy *et al.* 2003). Using the bovine α S1-casein promoter, it was found in mice that the –14 kbp promoter construct gave the highest level of expression in the mammary gland and was developmentally controlled according to the bovine rather than the mouse scheme (Rijnkels *et al.* 1998). The CoRE identified here at –10 kbp in the bovine α S1-casein promoter conceivably specifies one of the distal, highly relevant control elements contributing proper and strong lactation-dependent expression of the bovine α S1-casein gene.

The significance of the lactation-specific demethylation of this promoter area is easily explained with a wealth of evidence proving that promoter demethylation is associated with increased gene expression and that administration of hormones may cause decompaction of chromatin (Kascheike *et al.* 1997). Lactation-specific demethylation of casein-encoding genes has previously been demonstrated for mouse (Johnson *et al.* 1983). In this regard, we have not analysed the proximal promoter region of the α S1-casein promoter. However, considering the function-specific modulation of the methylation around the remote CoRE area as a paradigm for the entire gene, we would envisage that lactation-specific demethylation occurs also at other CpG dinucleotides dispersed over the gene. Indeed, function-associated demethylation of a proximal CpG dinucleotide in the bovine α S1-casein promoter (at position –774) has been observed during the analyses of transgenic mice lines, expressing a bovine α S1-casein promoter-driven lactoferrin-encoding transgene (Platenburg *et al.* 1996). Unclear, however, remains the significance of the observed increased percentage of methylated α S1-promoter molecules in the udders of these cows, which had given birth to calves but had never been milked. The two cows analysed in this regard had been sampled 10 and 14 days after calving. No histological specimen had been collected, but it is conceivable that the architecture of these udders

resembled those during involution, after the end of lactation. The udder is remodelled during this period and features a diminishing number of alveoli and MEC (cf. Molenaar *et al.* 2003, for histological images). Hence, the increased percentage of methylated promoter molecules measured in these samples might well have been caused by reasons unrelated to the absence of α S1-casein synthesis.

De novo methylation of the distal enhancer and chromatin condensation during acute down regulation of α S1-casein synthesis

We observed, within 24 h after infection, a reversion of the percentage of CpG-methylated α S1-casein promoter molecules from the lactation-specific level to that characterizing lactation competent, but not lactating udder tissue. The increased percentage of methylated α S1-promoter molecules in the infected tissues must be caused by *de novo* methylation in MEC. We excluded experimentally that neither a massive decrease in MEC nor increased numbers of immune cells recruited into the udder during infection can be the source for the increased percentage of methylated promoter molecules measured in the infected tissues. The observed lacking correlation of the degree of promoter methylation with the number of recruited immune cells is to be expected, if one compares the weights of the udder with that of the cells recruited into it during infection. The milked out udder weighs some 20 kg. MEC would contribute 1 kg if this cell type constitutes only 5% of the whole tissue from the fully lactating udder. On the other hand, the weight of immune cells in 20 l of milk may be calculated to amount to 10 g, if the milk contains 1×10^6 cells/ml. This calculation shows that increased numbers of immune cells inside the infected gland cannot be the source for the threefold increased percentage of methylated α S1-promoter molecules observed in whole extracts from the infected tissue.

Rapidity of the remethylation strongly suggests an acute regulatory significance, and we prove that this is mediated by compacting the chromatin within and around the CoRE. This insulates regionally in the infected tissue the enhancer region against the systemically unaltered high levels of lactation hormones as exemplified here for prolactin. Chromatin remodelling is a complex process involving a variety of different factors and enzymes, including also the regional deacetylation of histones H3 and H4. This process is known to eventually trigger *de novo* methylation of DNA (Strunnikova *et al.* 2005).

Molecular details of the mechanisms conveying the lactation-dependent regulation of the CpG-methylation status in this area of the α S1-casein promoter and the mastitis associated remethylation can only be analysed in lactation-competent MEC models. These are not available for the cow. Our *in vivo* model neither allows to experimentally dissect the sequence of events leading to *de novo* methylation, nor to analyse the specific role of STAT5 factors in this process (Jolivet *et al.* 2005). However, STAT5 factors have been shown to eventually recruit histone deacetylases (Xu *et al.* 2003).

Blunting of α S1-casein synthesis is caused by translational rather than transcriptional control mechanisms

We observed different quantitative extents and kinetics of the decay of α S1-mRNA and protein abundance during infection. The α S1-casein mRNA was reduced only to $\sim 50\%$ during the 24 h period of infection. Even this modest decrease indicates reduced or even blocked transcription, since it is long known that prolactin stabilizes *in vivo* the half-life of the α S1-casein mRNA more than 15-fold, to about 30–90 h, depending on the method of determination (Guyette *et al.* 1979, Rosen *et al.* 1980). Hence, fully lactating cows may maintain high concentrations of this mRNA species in the absence of transcription. On the other hand, the protein abundance remained high during an infection period of 12 h, albeit a steadily decreasing α S1-casein mRNA-concentration. The almost complete clearance of the udder tissue from both α S1- and β -casein within the period between 12 and 24 h post-infection is stunning. It occurred consistently in all four cows infected with *E. coli*. It can hardly be explained by more residual milk in the udder quarter infected for 12 h only, since all udder quarters had been milked out prior to sampling the tissues in a similar fashion. No signs were found for extensive general proteolysis or selective casein degradation in the 24 h infected samples, neither in the one- or two dimensional protein gels, nor in the western blots. Hence, the phenomenon clearly indicates that post-transcriptional control mechanisms for casein synthesis and/or stability are activated by this pathogenic strain of *E. coli*.

Our data together show that, in fully lactating cows, α S1-casein synthesis is blocked in a spatially restricted fashion on the transcriptional and translational level of gene expression. Translation of the α S1-casein mRNA is very effectively blocked by post-transcriptional control mechanisms, ensuring cessation of translation albeit prevailing high concentrations of the α S1-casein mRNA. Also these mechanisms operate spatially restricted, in the infected udder quarter alone. Sustained downregulation of gene transcription is achieved by promoter

remethylation. This induces compaction of the chromatin around the lactation-relevant enhancer of α S1-casein gene transcription, thereby insulating this genetic control element against the systemically unchanged high levels of lactational hormones. Our data attribute an acute regulatory significance to CpG methylation.

Acknowledgements

Part of this work contributed to the doctoral thesis of JH and WP. Mrs Angelika Deike, Maren Anders, Bärbel Pletz and Marlis Deutscher provided expert technical assistance.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (grants Se326/9-2 & 12-2). The European Community through the EADGENE Network of Excellence also supported this work. The authors declare that there exists no conflict of interests concerning this study that would prejudice its impartiality.

References

- Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P & Laible G 2003 Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. *Nature Biotechnology* **21** 157–162.
- Brunner JR 1981 Cow milk proteins: twenty years of progress. *Journal of Dairy Science* **64** 1038–1054.
- Bustin M 2001 Chromatin unfolding and activation by HMGN(*) chromosomal proteins. *Trends in Biochemical Sciences* **26** 431–437.
- Bühler ThA, Bruyère Th, Went DE, Stranzinger G & Bürki K 1990 Rabbit beta-casein promoter directs secretion of human interleucin-2 into the milk of transgenic rabbits. *Biotechnology* **8** 140–143.
- Cerdan MG, Young JI, Zino E, Falzone TL, Otero V, Torres HN & Rubinstein M 1998 Accurate spatial and temporal transgene expression driven by a 3.8-kilobase promoter of the bovine beta-casein gene in the lactating mouse mammary gland. *Molecular Reproduction and Development* **49** 236–245.
- Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng CX, Robinson GW & Hennighausen L 2004 Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Molecular and Cellular Biology* **24** 8037–8047.
- Doerfler W 2005 On the biological significance of DNA methylation. *Biochemistry (Mosc.)* **70** 505–524.
- Doppler W, Groner B & Ball RK 1989 Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat β -casein gene promoter constructs in mammary epithelial cell line. *PNAS* **86** 104–108.
- Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M & Bucher P 2001 DNA binding specificity of different STAT proteins. Comparison of *in vitro* specificity with natural target sites. *Journal of Biological Chemistry* **276** 6675–6688.
- Goldammer T, Zerbe H, Molenaar A, Schuberth HJ, Brunner RM, Kata SR & Seyfert HM 2004 Mastitis increases mammary mRNA abundance of {beta}-Defensin 5, Toll-Like-Receptor 2 (TLR2), and TLR4 but Not TLR9 in cattle. *Clinical and Diagnostic Laboratory Immunology* **11** 174–185.

- Gorg A, Weiss W & Dunn MJ 2004 Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4** 3665–3685.
- Gouilleux F, Wakao H, Mundt M & Groner B 1994 Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO Journal* **13** 4361–4369.
- Groenen MAM, Dijkhof RJM, van der Poel JJ, van Diggelen R & Verstege E 1992 Multiple octamer binding sites in the promoter region of the bovine α s2-casein gene. *Nucleic Acids Research* **20** 4311–4318.
- Groner B 2002 Transcription factor regulation in mammary epithelial cells. *Domestic Animal Endocrinology* **23** 25–32.
- Guyette WA, Matusik RJ & Rosen JM 1979 Prolactin-mediated transcriptional and posttranscriptional control of casein gene expression. *Cell* **17** 1013–1023.
- Hennighausen L, Robinson GW, Wagner K-U & Liu X 1997 Prolactin signaling in mammary gland development. *Journal of Biological Chemistry* **272** 7567–7569.
- Herrmann J 2001 On the significance of prolactin for milk formation in cattle: arrangement and biological significance of STAT5 DNA-binding sites in promoters of selected mammary gland expressed genes. *Dissertation, Faculty of Mathematics and Natural Sciences, Germany: University of Rostock*.
- Jaenisch R & Bird A 2003 Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* **33** (Suppl 1) 245–254.
- John S, Vinkemeier U, Soldaini E, Darnell JE Jr & Leonard WJ 1999 The significance of tetramerization in promoter recruitment by Stat5. *Molecular and Cellular Biology* **19** 1910–1918.
- Johnson ML, Levy J, Supowit SC, Yu-Lee LY & Rosen JM 1983 Tissue- and cell-specific casein gene expression II. Relationship to site-specific DNA methylation. *Journal of Biological Chemistry* **258** 10805–10811.
- Jolivet G, L'Hôte C, Pierre S, Tourkine N & Houdebine L-M 1996 A MGF/STAT5 binding site is necessary in the distal enhancer for high prolactin induction of transfected rabbit α s1-casein-CAT gene transcription. *FEBS Letters* **389** 257–262.
- Jolivet G, Pantano T & Houdebine LM 2005 Regulation by the extracellular matrix (ECM) of prolactin-induced alpha s1-casein gene expression in rabbit primary mammary cells: role of STAT5, C/EBP, and chromatin structure. *Journal of Cellular Biochemistry* **95** 313–327.
- Kascheike B, Ivell R & Walther N 1997 Alterations in the chromatin structure of the distal promoter region of the bovine oxytocin gene correlate with ovarian expression. *DNA and Cell Biology* **16** 1237–1248.
- Koczan D 1994 The bovine α S1-encoding gene: sequence, allelic variants, regulation of expression and biotechnological use. *Institute for Microbiology and Molecular Biology; Dissertation, Germany: University of Giessen*.
- Koczan D, Hobom G & Seyfert H-M 1991 Genomic organization of the bovine alpha-S1 casein gene. *Nucleic Acids Research* **19** 5591–5596.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Lechner J, Welte T, Tomasi JK, Bruno P, Cairns C, Gustafsson J-A & Doppler W 1997 Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of β -casein gene transcription. *Journal of Biological Chemistry* **272** 20954–20960.
- Lee K-F, DeMayo J, Aitee SH & Rosen JM 1988 Tissue-specific expression of rat beta-casein gene in transgenic mice. *Nucleic Acids Research* **16** 1027–1041.
- Lee CS, Kim K, Yu DY & Lee KK 1998 Pretreatment with glucocorticoid is essential for lactogenic induction of the bovine β -casein/CAT expression in HC11 cells. *Endocrine Research* **24** 65–77.
- Li E 2002 Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews Genetics* **3** 662–673.
- Litterst CM, Kliem S, Marilley D & Pfitzner E 2003 NCoA-1/SRC-1 is an essential coactivator of STAT5 that binds to the FDL motif in the [alpha]-helical region of the STAT5 transactivation domain. *Journal of Biological Chemistry* **278** 45340–45351.
- Liu X, Robinson GW, Wagner K-U, Garrett L, Wynshaw-Boris A & Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes and Development* **11** 179–186.
- Liu X, Gallego MI, Smith GH, Robinson GW & Hennighausen L 1998 Functional release of Stat5a-null mammary tissue through the activation of compensating signals including Stat5b. *Cell Growth and Differentiation* **9** 795–803.
- Mao J, Molenaar AJ, Wheeler TT & Seyfert H-M 2002 Stat5 binding contributes to lactational stimulation of promoter III expressing the bovine acetyl-CoA-carboxylase α -encoding gene in the mammary gland. *Journal of Molecular Endocrinology* **29** 73–88.
- McCabe DC & Caudill MA 2005 DNA methylation, genomic silencing, and links to nutrition and cancer. *Nutrition Reviews* **63** 183–195.
- Meade H, Gates L, Lacy E & Lonberg N 1990 Bovine α s1-casein gene sequences direct high level expression of active human urokinase in mouse milk. *Biotechnology* **8** 443–446.
- Molenaar A, Mao J, Oden K & Seyfert HM 2003 All three promoters of the acetyl-coenzyme a-carboxylase α -encoding gene are expressed in mammary epithelial cells of ruminants. *Journal of Histochemistry and Cytochemistry* **51** 1073–1081.
- Mukhopadhyay SS, Wyszomierski SL, Gronostajski RM & Rosen JM 2001 Differential interactions of specific nuclear factor I isoforms with the glucocorticoid receptor and STAT5 in the cooperative regulation of WAP gene transcription. *Molecular and Cellular Biology* **21** 6859–6869.
- Nan X, Ng H-H, Johnson CA, Laherty CD, Turner BM, Eisenman RN & Bird A 1998 Transcriptional repression by methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393** 386–389.
- Naylor MJ, Li N, Cheung J, Lowe ET, Lambert E, Marlow R, Wang P, Schatzmann F, Wintermantel T, Schuetz G *et al.* 2005 Ablation of β -1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *Journal of Cell Biology* **171** 717–728.
- Ng H-H & Bird A 1999 DNA methylation and chromatin modification. *Current Opinions on Genetics and Development* **9** 158–163.
- Paape MJ, Shafer-Weaver K, Capuco AV, Van Oostveldt K & Burvenich C 2000 Immune surveillance of mammary tissue by phagocytic cells. *Advances in Experimental Medicine and Biology* **480** 259–277.
- Pantano T, Jolivet G, Prince S, Menck-Le BC, Maeder C, Viglietta C, Rival S & Houdebine LM 2002 Effect of the rabbit alpha s1-casein gene distal enhancer on the expression of a reporter gene *in vitro* and *in vivo*. *Biochemical and Biophysical Research Communications* **290** 53–61.
- Pfitzner E, Jahne R, Wissler M, Stoecklin E & Groner B 1998 p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivating domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. *Molecular Endocrinology* **12** 1582–1593.
- Platenburg CJ, Vollebregt EJ, Karatzas CN, Kootwijk EP, De Boer HA & Strijker R 1996 Mammary gland-specific hypomethylation of Hpa II sites flanking the bovine alpha S1-casein gene. *Transgenic Research* **5** 421–431.
- Quandt K, Frech K, Karas H, Wingender E & Werner T 1995 MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Research* **23** 4878–4884.
- Rao S, Procko E & Shannon MF 2001 Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the *IL-2* gene. *Journal of Immunology* **167** 4494–4503.

- Raught B, Khursheed B, Kazansky A & Rosen J 1994 YY1 represses beta-casein gene expression by preventing the formation of a lactation-associated complex. *Molecular and Cellular Biology* **14** 1752–1763.
- Rijnkels M, Kooiman M, Krimpenfort PJA, De Boer HA & Pieper FR 1995 Expression analysis of the individual bovine, b, as2- and k-casein genes in transgenic mice. *Biochemical Journal* **311** 929–937.
- Rijnkels M, Kooiman PM, Platenburg GJ, van Dixhoorn M, Nuijens JH, De Boer HA & Pieper FR 1998 High-level expression of bovine alpha s1-casein in milk of transgenic mice. *Transgenic Research* **7** 5–14.
- Rosen JM, Matusik RJ, Richards DA, Gupta P & Rodgers JR 1980 Multihormonal regulation of casein gene expression at the transcriptional and posttranscriptional levels in the mammary gland. *Recent Progress in Hormone Research* **36** 157–192.
- Rosen JM, Wyszomierski SL & Hadsell D 1999 Regulation of milk protein gene expression. *Annual Review of Nutrition* **19** 407–436.
- Schindler U, Wu P, Rothe M, Brasseur M & McKnight StL 1995 Components of a Stat recognition code: evidence for two layers of molecular specificity. *Immunity* **2** 689–697.
- Schmidhauser C, Bissell M, Myers CA & Casperson GF 1990 Extracellular matrix and hormones transcriptionally regulate bovine β -casein 5' sequences in stably transfected mouse mammary cells. *PNAS* **87** 9118–9122.
- Schmitt-Ney M, Doppler W, Ball RK & Groner B 1991 Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. *Molecular and Cellular Biology* **11** 3745–3755.
- Seyfert H-M & Sawatzki G 1986 An estimation of the soluble tubulin content in Tetrahymena cells of normal and of size altered phenotype. *Experimental Cell Research* **162** 86–96.
- Seyfert H-M, Pitra C, Meyer L, Brunner RM, Wheeler TT, Molenaar AJ, McCracken JY, Hermann J, Thiesen H-J & Schwerin M 2000 Molecular characterization of STAT5A- and STAT5B-encoding genes reveals extended intragenic sequence homogeneity in cattle and mouse and different degrees of divergent evolution of various domains. *Journal of Molecular Evolution* **50** 550–561.
- Stöcklin E, Wissler M, Gouilleux F & Groner B 1996 Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* **383** 726–728.
- Strunnikova M, Schagdarsurengin U, Kehlen A, Garbe JC, Stampfer MR & Dammann R 2005 Chromatin inactivation precedes *de novo* DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter. *Molecular and Cellular Biology* **25** 3923–3933.
- Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G & Ihle JN 1998 Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine response. *Cell* **93** 841–850.
- Tomek W, Sterza FAM, Kubelka M, Wollenhaupt K, Torner H, Anger M & Kanitz W 2002 Regulation of translation during *in vitro* maturation of bovine oocytes: the role of MAP kinase, eIF4E (cap binding protein) phosphorylation, and eIF4E-BP1. *Biology of Reproduction* **66** 1274–1282.
- Ushijima T & Okochi-Takada E 2005 Aberrant methylations in cancer cells: where do they come from? *Cancer Science* **96** 206–211.
- Vanselow J, Pohland R & Furbass R 2005 Promoter-2-derived Cyp19 expression in bovine granulosa cells coincides with gene-specific DNA hypo-methylation. *Molecular and Cellular Endocrinology* **233** 57–64.
- Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J & Darnell JE Jr 1996 DNA binding of *in vitro* activated Stat1a, Stat1b and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. *EMBO Journal* **15** 5616–5626.
- Vonderhaar BK 1988 In *Breast Cancer: Cellular and Molecular Biology*, pp 251–266. Eds ME Lippman & RB Dickons. Boston: Kluwer.
- Vonderhaar B & Ziska SE 1989 Hormonal regulation of milk protein gene expression. *Annual Review in Physiology* **51** 641–652.
- Wheeler TT, Kuys YM, Broadhurst MM & Molenaar AJ 1997 Mammary Stat5 abundance and activity are not altered with lactation state in cows. *Molecular and Cellular Endocrinology* **133** 141–149.
- Wheeler TT, Broadhurst MK, Sadowski HB, Farr VC & Prosser CG 2001 Stat5 phosphorylation status and DNA-binding activity in the bovine and murine mammary glands. *Molecular and Cellular Endocrinology* **176** 39–48.
- Winklehner-Jennewein P, Geymayer S, Lechner J, Welte T, Hansson L, Geley S & Doppler W 1998 A distal enhancer region in the human β -casein gene mediates the response to prolactin and glucocorticoid hormones. *Gene* **218** 127–139.
- Wintermantel T, Schuetz G, Clarke AR, Mueller U, Hynes NE & Streuli CH 2005 Ablation of b-1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *Journal of Cell Biology* **171** 717–728.
- Wyszomierski SL & Rosen JM 2001 Cooperative effects of STAT5 (signal transducer and activator of transcription 5) and C/EBP β (CCAAT/enhancer-binding protein- β) on β -casein gene transcription are mediated by the glucocorticoid receptor. *Molecular Endocrinology* **15** 228–240.
- Xu M, Nie L, Kim SH & Sun XH 2003 STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBP β . *EMBO Journal* **22** 893–904.
- Yang W, Molenaar AJ, Kurts-Ebert B & Seyfert HM 2006 NF- κ B factors are essential, but not the switch, for pathogen-related induction of the bovine β -defensin 5-encoding gene in mammary epithelial cells. *Molecular Immunology* **43** 210–225.
- Yu-Lee L-Y, Luo G, Book ML & Morris SM 1998 Lactogenic hormone signal transduction. *Biology of Reproduction* **58** 295–301.

Received 30 June 2006
Accepted 21 August 2006