DNA-remethylation around a STAT5-binding enhancer in the αS1-casein promoter is associated with abrupt shutdown of αS1-casein synthesis during acute mastitis

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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 αS1-casein-encoding gene. This enhancer area is hypermethylated 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 ~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 αS1-casein mRNA concentration dropped to ~50% while the protein synthesis was shut down to ~2.5% in the infected quarters, alone. The methylation status of the promoter from a not lactationally regulated gene was unaltered, and the distal αS1-casein promoter was not remethylated in udder quarters with subclinical Staphylococcus aureus infections featuring sustained casein synthesis. Hence, infection-related remethylation of the αS1-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.

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Introduction

αS1-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 glucocorticoids 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, 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-coenzymeA-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 transcriptional 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, Wyszmierski & 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. 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 (Rijkenk 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) αS1-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 (Winternant et al. 2005), to cooperate not only with the glucocorticoid hormone receptor (GR; Stöcklin et al. 1996), mediated via C/EBP β (Wyszmierski & Rosen 2001), but also with the p300/CREB family of transcription-coactivators (Pfitzner et al. 1998). Both GR and p300/CREB interact yet with another coactivator, NcoA-1 (Litterst et al. 2003). The p300/CREB 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 αS1-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 αS1-casein mRNA, but at the same time express heavily immuned immune defense 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 aseptic conditions between 5 and 10 min after culling.

Washed udder skin was cut using an alcohol disinfected aseptical conditions. A small section of the udder quarter followed by the probe under aseptical conditions. A small section preparation of a 1 cm tissue piece from the centre of the udder quarter was taken out from a deeper area of the udder quarter followed by preparation of a 1 cm tissue piece from the centre of the probe under aseptic conditions. A small section (1 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 αS1-casein promoter

The distal αS1-casein promoter was retrieved from the λ phage λb2s1CNII, which had been isolated in the course of the initial description of the bovine αS1-casein-encoding gene (Koczyn et al. 1991, Koczyn 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 αS1-promoter containing reporter genes

The firefly luciferase expressing reporter gene harbouring the proximal promoter had been established in previous experiments (Koczyn 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 αS1-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′GCGGTTACCCAGGAAGAGTCTCTGAAAGATG, 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. Hybridon 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 usually 0·1× SSC and 65 °C (2×30 min). Radioactive signals on the filters were detected with the Storm 840 Phosphoimager (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′-TGAACTGTCGTAAGTCG and filled in 3′-P-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
the respective experimental reporter gene constructs with pSV2neo (Clonetech) 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 μg/ml ovine prolactin (Sigma) and 0·1 μM dexamethasone (Sigma; cf. Lee et al. 1998, Winklehner-Jennewein 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 αS1-encoding gene (positions −10 434 to −10 165) were 5′-TTGGTTAGGGGAATTTTGATTTATGTT (forward) and 5′-ATTCCAAAAATTCACACTATTCTT (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′-TGGAGAGTTTTTTTTGGGGAGGT (forward) and 5′-TCCCCAAAAATATACA-TTTAAAACCTCCT (reverse).

PCR was performed in 25 μl reaction volume with 0·25 U Taq polymerase (Qbiogene, Heidelberg, Germany). The first cycle was 94°C for 5 min, 53°C for 30 s, 70°C for 2 min. This was followed by 35–40 cycles of 94°C for 30 s, 53°C for 30 s, 70°C for 2 min. The last PCR step was 70°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'-AAGTGGTATAAGTAA-GATTGCTGATAG and TAAGTAAGGTGTGG-GTTTGTG for zS1-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 μl recommended buffer for restriction digestion with DdeI (Promega). A small aliquot (~10 μl) was removed to determine the DNA concentration. A volume containing 2 μg DNA was removed, filled up with restriction enzyme buffer to 150 μl. 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 μg 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 μl 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’-AACAATCCATGAC-CATCCTGAC; reverse AGGAAAGGATCTGAGTA-GTAG) amplified a 386 bp comprising segment of the zS1-casein with the STAT5-binding area of the zS1-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 αS1-casein and β-defensin mRNA**

The abundance of zS1-casein mRNA molecules was measured from TRIZOL (Invitrogen) extracted RNA samples, also using the LightCycler instrument (Roche). The oligonucleotide primers 5’-GTTTTG-GACAATTCTACCAGCT (forward) and 5’-CAT-AACTGTGAGATCGCCCTCAG 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’-AGGCCCT CAT-CACCTGCTCTT (forward) and 5’-GAAACAGGTCG-CAATCTGCTCTCT (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 amplificates 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 μl 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 μl 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 μA/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 analyzed 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 zS1-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 zS1-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 mM 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 zS1-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 retroposons 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 αS1-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±4.0%) of the DNA molecules from the liver tissue are methylated, but only 10.2±0.5% are methylated in the fully lactating gland. The values for the udders from the pubertal and not lactating glands are 42.2±2.8% and 36.3±1.5% respectively. These data show that lactation coincides with hypo-methylation of the CoRE in the αS1-casein promoter.

The remote STAT sites bind STAT5A and enhance lactational stimulation of αS1-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](https://example.com/figure2.png)

**Figure 2** The enhancer binds STAT5A and increases αS1-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 STAT5-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 αS1-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 αS1-casein promoter. We compared the potential of the glucocorticoid analogue dexamethasone and prolactin, alone and in combination, to induce the expression of two different αS1-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 αS1-casein expression during lactation.

Acute mastitis blocks casein synthesis and reduces αS1-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
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 αS1-casein from milk confirmed qualitatively a steep decrease of the αS1-casein content in the samples taken 24 h after infection (Fig. 3B). While the αS1-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 αS1-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 αS1-casein mRNA with the time of infection, from 143 ± 24 ± 10^6 copies down to 76-6 ± 5 × 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 αS1-mRNA abundance and protein synthesis indicates the operation of a strong translational control mechanism to shut down αS1-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.
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 αS1-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 simultaneously 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 αS1-casein promoter. The β-defensin promoters are highly compacted in the uninfected control quarters, whereas 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 αS1-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 αS1-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 αS1-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 αS1-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 αS1-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 1×10⁶ cell/ml of milk, while 5×10⁶ of cells per ml of milk were found in the animal featuring the least degree of αS1-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 αS1-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
infection. This quarter, but not both others, was clearly reprogrammed to immune defence, as indicated by a 15-fold increased expression of bacterialid 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 ×10⁶ cells per ml of milk respectively. However, the degree of methylation at the αS1-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 αS1-casein mRNA molecules in the three samples.

The data together show that the degree of methylation of the αS1-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 αS1-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 αS1-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 αS1-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 αS1-casein synthesis.

The detection of a distal enhancer in the far upstream region of the bovine αS1-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 αS1-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 αS1-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 αS1-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 signaling 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

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

<table>
<thead>
<tr>
<th>Udder quarter</th>
<th>% Methylation</th>
<th>Somatic cells (10⁵/ml)</th>
<th>αS1-mRNA copies (10⁶)</th>
<th>β-Defensin mRNA copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front left</td>
<td>10.2</td>
<td>34</td>
<td>367</td>
<td>1708</td>
</tr>
<tr>
<td>Front right</td>
<td>18.5</td>
<td>164</td>
<td>374</td>
<td>1076</td>
</tr>
<tr>
<td>Back left</td>
<td>18.4</td>
<td>1592</td>
<td>389</td>
<td>15005</td>
</tr>
</tbody>
</table>

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reconstitution system of STAT5-mediated prolactin signalling (Gouilleux et al. 1994). However, assigning a clear biological significance to a far distal CoRE in the αS1-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 αS1-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 αS1-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 αS1-casein promoter conceivably specifies one of the distal, highly relevant control elements contributing proper and strong lactation-dependent expression of the bovine αS1-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 αS1-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 αS1-casein promoter (at position −774) has been observed during the analyses of transgenic mouse lines, expressing a bovine αS1-casein promoter-driven lactoferrin-encoding transgene (Platenburg et al. 1996). Unclear, however, remains the significance of the observed increased percentage of methylated αS1-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 αS1-casein synthesis.

**De novo** methylation of the distal enhancer and chromatin condensation during acute down regulation of αS1-casein synthesis

We observed, within 24 h after infection, a reversion of the percentage of CpG-methylated αS1-casein promoter molecules from the lactation-specific level to that characterizing lactation competent, but not lactating udder tissue. The increased percentage of methylated αS1-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 × 10⁸ 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 αS1-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 remodulation 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 αS1-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 αS1-casein synthesis is caused by translational rather than transcriptional control mechanisms

We observed different quantitative extents and kinetics of the decay of αS1-mRNA and protein abundance during infection. The αS1-casein mRNA was reduced only to ~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 αS1-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 protein abundance remained high during an infection absence of transcription. On the other hand, the high concentrations of this mRNA species in the udder tissue from both αS1- 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, αS1-casein synthesis is blocked in a spatially restricted fashion on the transcriptional and translational level of gene expression. Translation of the αS1-casein mRNA is very effectively blocked by post-transcriptional control mechanisms, ensuring cessation of translation albeit prevailing high concentrations of the αS1-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 αS1-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.

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