STAT5 binding contributes to lactational stimulation of promoter III expressing the bovine acetyl-CoA carboxylase α-encoding gene in the mammary gland

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Abstract

Activity of acetyl-CoA carboxylase (ACC-α) is rate limiting for de novo synthesis of fatty acids. The encoding gene is expressed by three different promoters. We characterized promoter III (PIII) from cow, previously only known from sheep. Quantitation of transcripts by RNase protection assays and real time PCR revealed that PIII is primarily expressed and strongly induced (−28-fold) in the lactating mammary gland. PIII transcripts are expressed in mammary epithelial cells (MEC) as shown by in situ hybridization. A 2999 bp segment of the PIII promoter conferred prolactin and dexamethasone inducibility to a luciferase reporter gene in stably transfected mouse MEC cells. Lactogenic induction was abolished if a unique signal transducer and activator of transcription (STAT)-binding site at position −797 was inactivated by two point mutations. An oligonucleotide probe harboring this STAT-site specifically bound nuclear proteins from the lactating mammary gland. Binding was abolished by those two point mutations and super-shift analyses showed that STAT5A factors are present in this complex. Hence, prolactin, acting through STAT5, contributes to the activation of ACC expression in the milk producing cells of the lactating mammary gland. We discuss that STAT5 might be important in determining the milk composition by coordinating fatty acid and protein synthesis during lactation.

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Introduction

The rate-limiting step in the formation of long chain fatty acids of all organisms is the carboxylation of acetyl-CoA to form malonyl-CoA, which is catalyzed by the enzyme acetyl-CoA carboxylase-α (ACC-α; E.C.6·4·1·2; Wakil et al. 1983). Fatty acids are required in all cells for membrane formation, but are also used in specialized tissues for fat synthesis either to be stored in adipose tissue or to be secreted into milk by the mammary gland. The role of ACC-α in milk fat synthesis primed our interest to study this enzyme from cattle, specifically to address the role of ACC-α in determining the relative amounts of fat and protein in milk.

Regulation of the enzymatic activity of ACC-α is very complex, due to the different demands of various cell types for fatty acid synthesis (Kim 1997). The ACC-α enzyme is very large (2346 amino acid (aa) residues), and multimerizes to exert its function. The enzyme activity is regulated at various levels, including short term regulation via allosteric interactions with metabolites (Thampy & Wakil 1988, Kim 1997) and reversible phosphorylation (Hardie 1989). Long term regulation involves multiple hormonal and nutritional controls of ACC-α gene expression (Iritani 1992).

Tissue-specific expression of the ACC-α-encoding gene in human (Ha et al. 1994), rat (Lopez-Casillas et al. 1989, 1991) and chicken (El Khadir-Mounier et al. 1996) is under the control of two different promoters (PI and PII). Their alternative usage results in heterogenous exon compositions of the 5’- untranslated region (UTR) of mRNA.
(Lopez-Casillas et al. 1989). Exon 5 contains the translational start codon in transcripts generated by both of these promoters. PI transcripts are principally expressed in adipose tissue, but are repressed in this tissue during lactation (Lopez-Casillas et al. 1991). PI activity is under tight nutritional control in the liver, since feeding a fat-free diet to fasted animals strongly induces the otherwise repressed promoter (Lopez-Casillas et al. 1991). PII is considered the housekeeping promoter, and contains an elaborate array of cis-regulatory elements that affect its activity (Luo & Kim 1990). PII is also lactationally stimulated in the mammary gland of the rat (Lopez-Casillas et al. 1991) and is presumably important for the generation of milk fat in this species. Both chicken promoters were shown to be regulated by thyroid hormone, glucagon and medium-chain fatty acid (Yin et al. 2000, Zhang et al. 2001).

A third promoter of the ACC-α-encoding gene (PIII) has been identified in sheep (Travers & Barber 1997, 1998). PIII transcribes the mRNA from exon 5A, which is located between exons 5 and 6. Activity of PIII leads to the formation of an alternative isoform of ACC-α, in which 17 aa residues encoded by exon 5A substitute for the N-terminal 75 aa residues. PIII was found to be expressed in several tissues, including kidney, liver, lung and quite strongly in the mammary gland. The physiological implications of the N-terminal alteration of the enzyme are still unknown. Lactation stimulates expression of promoter PIII in the mammary gland about 15-fold (Barber & Travers 1998). This observation raised our interest to study PIII from cattle, since its strong lactational induction suggests that this promoter might be important for milk fat formation.

An intriguing question regarding the molecular mechanisms controlling milk fat synthesis is how this is balanced with milk protein synthesis. In dairy cattle the ratio of milk protein/milk fat is in part genetically inherited (Hayes et al. 1984, Dematawewa & Berger 1998). However, the molecular mechanisms determining this are largely unknown. Several lines of evidence suggest that the lactational hormone prolactin might be crucial for this coordination.

Prolactin is important for development of the mammary gland and lactation (Vonderhaar 1987, 1988, Vonderhaar & Ziska 1989, Hennighausen et al. 1997, Liu et al. 1997). Prolactin, synergizing with glucocorticoid hormone was shown to be required for eliciting casein synthesis in vivo (Guyette et al. 1979, Rosen et al. 1980) and these hormones activate β-casein expression in model cells (Doppler et al. 1989). Indeed, the relevance of prolactin signaling for the stimulation of milk protein synthesis (e.g. caseins, β-lactoglobulin) has led to the identification of the latent transcription factor STAT5 (Gouilleux et al. 1994, Hennighausen et al. 1997, Liu et al. 1997), originally known as mammary gland factor (MGF, Schmitt-Ney et al. 1992, Welte et al. 1994, Wakao et al. 1994). STAT5 was subsequently shown to consist of two highly related factors, STAT5A and STAT5B (Liu et al. 1995). They transmit the signals of a variety of cytokines and peptide hormones, among them prolactin. STAT5 factors, like the other STAT transcription factors, are activated by phosphorylation mediated by JAK2 kinase. JAK2 kinases, in turn, are activated by hormone receptors after these have bound their appropriate hormonal ligand. Once phosphorylated, STAT5 factors homo- or heterodimerize, translocate into the nucleus and bind to promoters containing STAT factor binding sites. The consensus sequence for all STAT factors (except for STAT6) is 5'-TTCCNN NGAA (Schindler et al. 1995, Ehret et al. 2001). It is widely accepted that prolactin signaling mediated by STAT5 factor binding to target promoters, is necessary for the expression of the genes encoding milk proteins (Watson & Burdon 1996, Wyszomierski et al. 1999, Gallego et al. 2001). The role of prolactin signaling in inducing synthesis of fatty acids in the lactating mammary gland in general and within mammary epithelial cells in particular has not yet been studied at the molecular level.

Materials and Methods

Rapid amplification of 5'-cDNA ends (RACE)-PCR

The sequence of exon 5A of the bovine ACC-α-encoding gene was obtained by 5'-RACE. These experiments were carried out using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). Double-stranded cDNA was synthesized using 2 μg bovine mammary gland total RNA primed with gene-specific primer Ac_ex6–7r (5'-TGGCAATGAGAACCTTCTCA

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ATC) spanning bovine ACC-α exons 6 and 7. cDNA copies were ligated to the adaptor, amplified with the forward adaptor primer (supplied with the kit) together with the gene-specific primer Ac_ex6rm (5'-CAAATCTCTGGAGAGGCTACA). RACE products were cloned into pGEM-T Easy (Promega) and sequenced.

Preparation of RNA and synthesis of the first strand cDNA

Total RNA from different cow tissues was prepared using the guanidium thiocyanate/cesium chloride procedure as described (Chirgwin et al. 1979). The first strand cDNA was synthesized with SuperScript II RNaseH- Reverse Transcriptase (Life Technologies, Inc.). Two micrograms total RNA and 20 pmol random hexamer primers (Clontech) were heated at 70 °C for 10 min, chilled on ice, and reverse transcribed in a total volume of 20 µl of a buffer, which contained 50 mM Tris–HCl, pH 8·3, 77 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0·5 mM of each of the dNTPs. The reaction was incubated at 45 °C for 1 h and terminated by heating at 95 °C for 5 min.

RT-PCR

One microliter of the cDNA corresponding to 100 ng total RNA was used as template for subsequent PCR amplifications. Assays (25 µl) contained PCR-incubation buffer (10 mM Tris–HCl, pH 9·0; 50 mM KCl; 1·5 mM MgCl₂; 0·1% Triton X-100; 0·2 mg/ml BSA or gelatin), 200 µM dNTPs, 10–25 pmol of each forward and reverse primer, and 0·25 units of Taq DNA polymerase (Appligene-Oncor, Heidelberg, Germany). ‘Touchdown’ amplification protocols were used throughout (Don et al. 1991). An initial denaturation (4 min at 94 °C) preceded 10 cycles in which the annealing temperature was lowered from 70 to 60 °C in 1 °C decrements. These were followed by 20–30 standard cycles (1 min at 94 °C, 1 min at 60 °C and 3 min at 70 °C). After the final cycle, samples were extended at 70 °C for an extra 10 min.

Cloning of PIII and exon 5A of the bovine ACC-α-encoding gene

The genomic region harboring PIII of the bovine ACC-α-encoding gene was isolated from Genome Walker Libraries, established with the Universal GenomeWalker Kit (Clontech) and our bovine bacterial artificial chromosome clone BAC91 (Mao et al. 2001) as template. This clone contains the entire promoter region of the bovine ACC-α-encoding gene. Separate aliquots of BAC91 DNA were digested with EcoRV, ScaI, StuI, Pvull, Drai, respectively. The fragments were ligated with the GenomeWalker Adaptor, generating five libraries. Each individual library was amplified with forward adaptor primer 1 of the kit and the gene-specific reverse primer Ac_5Ar1 (5'-TCTCTTCAGCTGGCCTTG) using the Expand Long Template Amplification Kit (Roche). PCR products were diluted 50-fold and 1 µl used as template in the secondary PCR with the forward adaptor primer 2 of the kit and the nested gene-specific reverse primer Ac_5Ar2 (5'-CCACACAGCATCAGCAGATTTC). A 3·2 kb fragment was obtained from the BAC91-EcoRV library. This fragment was digested with Klenow enzyme to obtain blunt ends, then digested with SalI and cloned into the SalI/SmaI site of pBluescript KS+ (Stratagene), resulting in clone PIII-3·2 kb. The promoter sequence was obtained from both strands using automated ABI 310 (Applied Biosystems, Forster City, CA, USA) or LICOR4000L (MWG-Biotech, Ebersberg, Germany) DNA sequencers.

Primer extension

Primer extension was performed as described (Henke et al. 1996). Briefly, 1 pmol of extension primer was end-labeled with 32P using T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol). It was annealed with 10 µg total RNA and transcribed in reverse using 100 units of SuperScript II RNase H- Reverse Transcriptase (Life Technologies, Inc.) for 1 h at 45 °C in a total volume of 20 µl. Extension products were analyzed on a 6% polyacrylamide/8 M urea sequencing gel, next to the sequencing ladder of the corresponding subcloned genomic DNA. For sequencing, the dsDNA Cycle Sequencing System (Life Technologies, Inc.) and the same radiolabeled extension primers were used.

RNase protection assays

RNase protection assays were performed essentially as previously described (Gilman 1993). Labeled antisense RNA probes were generated from
linearized plasmids containing exon 5 (253 bp) of the bovine ACC-α gene, exon 5A (474 bp) of the bovine ACC gene and a 206 bp segment of the bovine β-actin cDNA (nt 211–417, GenBank accession number K00622) using [α]32P]rCTP (800 Ci/mmol, Amersham) and T7 RNA polymerase (Promega). The specific radioactivity of the actin probe was reduced by adding unlabeled rCTP to the reaction at two-thirds the concentration of the other ribonucleotides. The probes were gel purified before use. Hybridization was carried out overnight at 65 °C using a total of 40 µg RNA and 50 000 c.p.m. of each probe. RNA that was protected from the subsequent RNase A/RNase T1 digestion was resolved on a urea–acrylamide gel containing 5% (w/v) acrylamide and 0·17% (w/v) bis-acrylamide. The protected RNA fragments were visualized by exposure to X-ray film.

**Real time RCR quantitation of ACC-α transcripts**

Real time PCR with a light cycler (Roche) was used to quantify the abundance of transcripts derived from PIII in various tissues. The abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also measured in these RNA preparations. All amplification primers were placed on different exons. For first strand cDNA synthesis 1 µg total RNA was doubly primed with 10 pmol oligo-dT and 10 pmol of the ACC-α-specific reverse primer Ac-990r (5’-TTTCAGAGAAGTTCTGGG-AGCT), located on exon 9 (Mao et al. 2001). After first strand synthesis of the cDNA (Superscript, H-, Life Technologies) products were purified (High Pure PCR Product Purification Kit, Roche) and aliquots equivalent to 12·5 ng total RNA distributed onto different vials. A 225 bp segment was amplified from the PIII-derived transcripts, using the forward primer Ac_ex5Af 5’-CTACTCTGAACTGAGGTCCTCA and the reverse primer Ac_ex5Ar 6r (5’-GCCAGACAT GCCGAGATCTTTG), bridging the splice boundary between exons 5A and 6.

GAPDH messages were amplified with the primers 5’-CATGGACCTTCTACTAGGCT and 5’-ACCTTAAAGTGAGCCCGA (forward and reverse respectively). Assays were run with the LightCycler-FastStart DNA Master Syber GreenI kit (Roche), essentially as described in Mao et al. (2001). All samples were measured in duplicate. A dilution series (10⁶ to 10² copies) of appropriate cDNA subclones was included in each run and served to calibrate the results for number of copies of transcripts. Values from different individuals were normalized within tissues for equal GAPDH copy numbers.

**Construction of reporter plasmids and site-directed mutagenesis of the STAT-binding site in PIII**

A 3·0 kb fragment was excised from plasmid PIII-3·2 kb using the KpnI site in the linker of the cloning vector and the PvuII site at position +170 in exon 5A. This fragment was cloned into KpnI/SmaI sites of the promoterless luciferase reporter gene vector pGL3 basic (Promega), generating clone PIII (−2999). To delete the STAT-binding site in PIII, a 51 bp fragment between positions −799 and −749 was deleted. Therefore, a 2·2 kb segment of PIII was amplified from the BAC91/EcoRV genome walker library using adopter primer AP2 as forward primer and the oligo nucleotide P3st5dr (5’-CCCTTCAAAC AGAAGTACACAA, positions −800 to −822 of the promoter) as reverse primer. The amplified fragment was treated with Klenow enzyme and digested with Sall. This fragment was ligated into clone −2999 in which a Sall/HpaI fragment had been deleted, exploiting the unique HpaI cutting site at −749 of the promoter (clone Del). Two point mutations were introduced into the STAT-binding motif (TTGTTGGA) of construct PIII (−2999) using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega), essentially as described in the kit. A mutation oligonucleotide (5’-GAAGGGTTTTTGTGATGTATTTATAG ATGTCCGGCAA) was used to introduce point mutations to generate clone M1. The expression plasmids were verified by sequencing.

**Cell culture, transfection and hormone induction**

Murine mammary epithelial HC11 cells were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 µg/ml insulin, 1 ng/ml epidermal growth factor (EGF), and 50 µg/ml gentamicin as previously described (Doppler et al. 1989).
For transient transfections, cells were split into 24-well plates (5 × 10^4 cells per well) 1 day prior to transfection. Five hundred nanograms of plasmid containing luciferase reporter gene and 10 ng of the control plasmid pCMV-β-gal (Stratagene) were co-transfected using LipofectAMINE PLUS (Life Technologies, Inc), according to the manufacturer’s instructions. The transfection mixture of LipofectAMINE-PLUS and plasmid DNA was replaced with fresh medium containing 10% FCS after 3 h incubation.

To generate pools of stably transfected HC11 cells, 4 µg of the indicated reporter gene construct, and 1 µg pSV2 neo (Clontech) were co-transfected, per 10 cm plate. Two weeks later, pools of G418-resistant colonies (>500) were selected. Prior to hormone induction, HC11 cells were grown to confluency in medium without insulin and kept confluent for 2 days. EGF was washed out and cells were cultured for 2 days in EGF-free medium, containing 5% FCS, insulin, 5 µg/ml ovine prolactin (Sigma) and 0·1 mM dexamethasone (Sigma) (Lee et al. 1998, Winklehner-Jennewein et al. 1998).

**Reporter gene assays**

Luciferase and β-galactosidase activities were measured in one-tube assays from cell extracts. The cells were washed with ice-cold phosphate-buffered saline, scraped into 50 µl lysis buffer (100 mM potassium phosphate (pH 7·8), 0·2% Triton X-100 and 0·5 mM dithiothreitol), centrifuged at 11 000 g for 5 min at 4 °C and supernatants removed by aspiration. Aliquots of the supernatant were assayed for luciferase and β-galactosidase activities with the Dual Light Chemiluminescent Reporter Gene assay system (Tropix, Bedford, MA, USA) as described in the manufacturer’s instructions. Differences in the individual transfection efficiencies were compensated for by normalizing the luciferase activities to a constant β-galactosidase activity. In each experiment, each construct was transfected in triplicate and the experiments were repeated at least three times. Luciferase activities from pools of stably transfected HC11 cells were normalized according to the protein concentrations of the extracts, as measured using the Bradford reagent protein assay kit (BioRad Laboratories).

**Electrophoretic mobility shift assay (EMSA)**

**Extract preparation**

Nuclear extracts of mammary tissues from lactating cows were performed essentially as described (Wheeler et al. 1997). Ground frozen bovine tissue (0·4 g) was homogenized in 10 ml low salt (LS) buffer (10 mM Heps pH 7·9, 1·5 mM MgCl₂, 10 mM KCl, 0·5 mM DTT, 0·2 mM PMSF and 1 mM sodium orthovanadate) containing 0·5% (v/v) NP-40. The homogenate was filtered through cheesecloth. Samples were kept on ice throughout the procedure. Nuclei and cell debris were pelleted by centrifugation (1000 g, 10 min), resuspended and washed in 1 ml LS buffer without NP-40. The final pellet was resuspended in a minimal volume of LS buffer (~ 0·1 ml). An equal volume of high salt (HS) buffer (20 mM Heps (pH 7·9), 0·6 M KCl, 1·5 mM MgCl₂, 0·5 mM DTT, 25% (v/v) glycerol) was added dropwise to the suspension with constant mixing. The solution was mixed end-over-end for 30 min, centrifuged at 10 000 g for 10 min, and the supernatant was dialysed for 5 h against buffer D (20 mM Heps (pH 7·9), 0·1 M KCl, 0·2 mM EDTA, 20% (v/v) glycerol) (Dignam et al. 1983). Aliquots of the dialyzed extracts were snap frozen in liquid nitrogen and stored at −70 °C. Protein concentrations were measured using the Bradford reagent protein assay kit (BioRad Laboratories).

**Probe generation**

A Klenow fill-in procedure was used to generate 32P-labeled double-stranded oligonucleotide probes for band shift analyses. The oligonucleotide 5′-GTGGGATTCCAAATTGATGGGAATGT (STAT binding site underlined) was annealed with a short reverse primer (5′-ACATCCACCGA) and labeled for 10 min in the presence of 20 µCi [α-32P]dCTP using the Mega-Prime kit (Amersham). After 30 min, supplementary unlabeled dCTP was added to ensure that the reaction proceeded to completion. Unlabeled double stranded competitor oligonucleotides were generated basically by the same technique, omitting the radiolabeled dCTP.

**Complex formation and resolution**

DNA–protein binding reaction mixtures (20 µl) contained 2–4 µg nuclear extracts, 1 µg poly[dI-dC] and 10 µl 2 × binding reaction buffer (20 mM
A) Genomic Organization of Promoters

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<th>3</th>
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<th>5</th>
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RT-PCR

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B) Definition of PIII

Ad LMG T G C A

[bp] 1,230 1,033
[bp] 653 517 453 394

754 bp 575 bp

M LI AD KI BR MU LU MG LMG

517 394
Hepes (pH 7.9), 10 mM MgCl₂, 0.2 mM EDTA, 2 mM DTT, 100 mM NaCl, 0.4 mM PMSF, 20% (v/v) glycerol). Reactions were incubated at room temperature for 10 min. Competitor oligonucleotides were included in three different concentrations representing 100-, 50- or 25-fold molar excess of the probe. Their sequence conformed either to the wild-type sequence or to that mutation, as introduced into the expression construct. Thus, the sequence 5’-GTTTTGAAGG GTTTTGGTTATGT served to synthesize double stranded mutated competitor oligonucleotides in which the STAT-binding site is altered by the same two point mutations (bold letters) as used in the expression construct M1. Supershift analyses contained anti-STAT5 A antibodies [Santa Cruz (Santa Cruz, CA, USA) sc-1081; 1 µg/assay]. Next, 10–40 fmol of the labeled probe were added and the mixture incubated for a further 20 min at room temperature. Samples were subjected to electrophoresis through 5% polyacrylamide gels (30:1 acrylamide–bisacrylamide) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Radiolabeled protein–DNA complexes were detected using a STORM-PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

In situ hybridization

In situ hybridization was performed essentially as described (Molenaar et al., 1992). A 474 bp comprising segment of exon 5A was amplified from template BAC91 DNA with the primers 5’-GTGA CCCTCAGAAGAAAGTGCAC and 5’-GCCCTCTAACACTTACCTTTGA (forward and reverse respectively). The amplified product was cloned into pGEM-T Easy and verified by sequencing. Thus, the sequence 5’-TCGTTTTGAAGG GTTTTGGTTATGT served to synthesize double stranded mutated competitor oligonucleotides in which the STAT-binding site is altered by the same two point mutations (bold letters) as used in the expression construct M1. Supershift analyses contained anti-STAT5 A antibodies [Santa Cruz (Santa Cruz, CA, USA) sc-1081; 1 µg/assay]. Next, 10–40 fmol of the labeled probe were added and the mixture incubated for a further 20 min at room temperature. Samples were subjected to electrophoresis through 5% polyacrylamide gels (30:1 acrylamide–bisacrylamide) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Radiolabeled protein–DNA complexes were detected using a STORM-PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Results

Isolation and definition of bovine PIII and exon 5A

Retrieval and basic characterization of the entire genomic region containing the three bovine ACC-α promoters has previously been described in detail (Mao et al., 2001). Briefly, promoter III (PIII) was identified based on 5’-RACE amplifications using mRNA from the lactating mammary gland. These experiments yielded several clones containing bovine exon 5A, as identified by the high degree of sequence similarity (96.5% over 441 bp) of the bovine exon 5A to its homolog from sheep (Barber & Travers, 1998), encoding an identical N-terminal alteration of the amino acid sequence of the enzyme as reported there. Nested reverse primers derived from the bovine exon 5A sequence were used to isolate PIII from Genome Walker libraries obtained from the BAC clone 91 containing the entire 5’-region of the bovine ACC-α-encoding gene (Mao et al., 2001). More than 3 kbp of PIII and exon 5A were subcloned, sequenced and used to establish promoter expression constructs. Long-span PCR amplifications determined the location of PIII relative to exons 5 and 6 (Fig. 1A).

Primer extension analyses combined with RT-PCR verified the expression of transcripts from PIII in mammary gland and adipose tissues (Fig. 1B). PIII is a TATA-boxless promoter initiating transcription from three different starts. We define

Figure 1 Definition of promoter III of the bovine ACC-α-encoding gene. (A) Genomic distribution of the three promoters (PI, PII, PIII) and the 5’-terminal 6 exons of the gene. Exon sizes are indicated and also the approximate distances between exons, as revealed by long span PCR amplifications using BAC91 as template (Mao et al., 2001). The sequence data of exon 5A and PIII is deposited as EMBL file AJ312201. (B) Primer extension analysis of PIII. Ten micrograms total RNA from adipose tissue (Ad) or lactating mammary gland (LMG) were primed with the 32P end-labeled oligonucleotide primer (Ac_5Ar2, cf Fig. 2). The three extension products (s1, s2, s3) and the complementary nucleotide sequence around s1 and s2 are shown (asterisks, complementary nucleotides to tsp1 and 2). Tsp1 (s1) was defined as position +1. Upper right panel shows the results of RT-PCR amplification of the PIII-derived mRNA from mammary RNA using primer AcP3f1 (immediately downstream of s3) and reverse primers annealing either to exon 5A (lane 1) or exon 6 (lane 2). Lower right panel shows RT-PCR amplifications using RNAs from various tissues as templates (L, liver; AD, adipose tissue; KI, kidney; BR, brain; MU, muscle; LU, lung; MG, pubertal mammary gland; LMG, lactating mammary gland; M, size marker). The open arrow denotes a splice variant seen in the adipose tissue only.
the most 3′-located transcriptional start site (s1; Figs 1B and 2) as position +1, hence 71 bp upstream of the previously identified transcriptional start point (tsp) of ovine PIII (Barber & Travers 1998). The bovine promoter sequence conforms to 92.7% (over 1477 bp) of the ovine homologue. Transcripts generated from PIII are primarily expressed in the lactating mammary gland.

RT-PCR amplifications with an exon 5A-derived forward primer and a reverse primer anchored on exon 6 demonstrated the presence of transcripts from PIII in a variety of bovine tissues including liver, adipose tissues, kidney, brain, muscle, lung, pubertal and lactating mammary gland. The results of RT-PCR amplifications are shown in the panels to the right of Fig. 1B. The products were of the expected size, and subcloning and sequencing verified their authenticity. From the adipose tissue, a shorter variant was also obtained. Sequence analysis revealed that in this variant 117 bp are deleted from the 5′-UTR encoded by exon 5A, exploiting kryptic splice donor and acceptor sites.
(Fig. 2). It contributes only a minor fraction of all PIII derived transcripts from this tissue, as revealed by real time RT-PCR control experiments (see below).

Lactation-specific differences in the expression of PIII in the mammary gland were first examined qualitatively in RNase protection assays (Fig. 3A). The signal intensities obtained with two of the three lactating mammary glands were much stronger than those from non-lactating, pregnant cows. However, absence of an exon 5A related-signal in the third lactating mammary gland indicates a high degree of individual variability of PIII expression at full lactation (Fig. 3A, compare lanes 4 and 5 with lane 6). In contrast, the same RNA samples revealed a much smaller lactation-associated difference in signal intensities, if an exon 5-specific probe was used (Fig. 3B). Such transcripts are generated by the activity of either PI or PII.

Real time RT-PCR was used to quantify the level of expression from PIII in various tissues, based on a different set of animals than that used for RNase protection assays. We compared expression of PIII in mammary gland, lung, liver, subcutaneous adipose tissue and muscle (Fig. 3C) from each of five lactating cows and virgin, pubertal females. Expression from PIII was highest in the lactating mammary gland (>3500 copies per RNA equivalent). The individual variability was very large, as was already evident from the RNase protection assay. However, the average concentration of transcripts was much (10- to >50-fold) higher in the lactating mammary gland than in any other tissue examined. Moreover, expression of PIII in the mammary gland is strongly and significantly (t test, \( P<0.02 \)) induced during lactation. The average concentration of PIII-derived transcripts is about 28-fold higher in total RNA from the lactating gland than that measured in glands from virgin, pubertal cows. The amount of all ACC-\( \alpha \) transcripts is about three-fold higher in the lactating mammary gland than in the pubertal gland (Mao et al. 2001). Thus, the fraction of PIII-derived transcripts from the total number of ACC-\( \alpha \)-encoding transcripts (reference values in Mao et al. 2001) increases more than 10-fold with lactation, from an apparent 0.7% in the pubertal gland to 8% at full lactation. This fraction is much smaller in all other tissues examined, less than 5% in liver and lung of lactating animals and 1% or less in all other tissues. Thus, the pattern of differential expression of PIII in the cow conforms essentially to that of sheep (Barber & Travers 1998).

Real time PCR was also used to compare in adipose tissue the proportion of spliced exon 5A transcripts to those of the total length (cf. Fig. 2). Using another group of six lactating cows we measured the copy numbers either with primer Ac_5 Afl (starting at position +36) or Ac_5Af, located on the spliced out segment of exon 5A. Both primers yield indistinguishable mean copy numbers (69 ± 21 and 68 ± 20, respectively), as measured with the same reverse primer (Ac_5 Aex6r). Hence, the splice variant contributes only very little to the total amount of PIII derived transcripts in adipose tissue. We measured also the abundances of both transcripts in the lactating mammary glands of the same animals as control and found no significant difference (2279 ± 322 and 2390 ± 515 respectively). Taken together, these data confirm also that the abundance of PIII-derived transcripts is much smaller in adipose tissue than in the lactating mammary gland. This was not evident from the initial primer extension experiment (Fig. 1). However, the particular RNA preparations used for the primer extension experiment had not been analysed for the abundance of PIII-derived transcripts, which eventually may be quite low in some lactating mammary glands, as shown above (cf. Fig. 3A and C).

**PIII is expressed in mammary epithelial cells**

In situ hybridizations were performed using an exon 5A antisense RNA to probe sections of mammary glands from pregnant or lactating cows. Strong hybridization signals were seen in the mammary epithelial cells (MECs) lining the alveoli (Fig. 4). Therefore, MECs are the major site of PIII-derived transcripts within the gland. Hybridizations with an \( \alpha \)S1-casein probe in serial sections detected the same areas, demonstrating that PIII-expressing alveoli were also highly expressing the \( \alpha \)S1-casein-encoding gene (not shown). Significantly, very little, if any, PIII-related hybridization signals are detected in areas of stromal and adipose tissue. Thus, most of the exon 5A-encoding mRNA in mammary tissue is derived from PIII activity within the mammary epithelial cells.
Figure 3  PIII is strongly expressed in the lactating mammary gland. (A) Quantification of PIII ACC transcripts using the RNase protection assay. The assays shown in lanes 1–6 contained 40 µg RNA isolated from mammary tissue from three pregnant and three lactating cows. Signals emerge only if RNA is added and their intensity increases with the amount of RNA added (lanes 7–9). Lanes 10 and 11 show the position of the fragment which is protected by the exon 5A-specific probe. The assays shown in lanes 1–9 contained both ACC PIII and β-actin probes, whereas those shown in lanes 10 and 11 contained only the PIII probe. The assays shown in lanes 8, 9 and 11 used the same mammary RNA as the assay in lane 4. Arrows indicate the protected fragments corresponding to the PIII and β-actin transcripts. (B) Quantification of PI/II ACC transcripts using the RNase protection assay. The assays shown in lanes 1–6 were performed using 40 µg RNA isolated from mammary tissue from the same three pregnant and three lactating cows as described above. All the assays contained both the ACC PI/II and β-actin probes. Signals emerge only if RNA is added (lanes 7–9). Arrows to the left of the panel indicate the positions of the protected fragments corresponding to the ACC PI/II and β-actin transcripts, while those to the right show the positions of the undigested probe molecules. (C) Real time PCR quantification of PIII-derived transcripts. Total RNA was extracted from various tissues (MG, mammary gland; LI, liver; LU, lung; AD, subcutaneous adipose tissue; MU, muscle), collected from five lactating cows (hatched columns) and five virgin, pubertal female animals (open columns) and assayed for the concentration of transcripts containing exon 5A. Copy numbers were determined on the basis of a dilution series of a cDNA subclone containing exons 5A to 9. Tissue-specific values from different individuals were normalized according to the simultaneously recorded GAPDH copy numbers. The mean values together with the S.E.M. (error bars) are presented.
Prolactin induction of PIII requires integrity of a STAT site at −797

We used a 2999 bp segment of PIII to examine the capacity of this promoter to drive luciferase expression in the established murine MEC line HC11. Pilot experiments (not shown) revealed in transient transfections \( (n=6) \), that this construct drives reporter gene expression strongly in HC11 cells (33·9 ± 2·8-fold higher than the promoterless luciferase vector pGL3 basic).

Induction of the promoter by the lactogenic hormones prolactin and dexamethasone was assessed in pools of stably transfected HC11 cells. Combined administration of these hormones induces transcription from the 3 kbp promoter segment of PIII by 3·5-fold on average \( (n=13; \text{S.E.M.}=0·4) \). A consensus STAT-binding sequence is present at position −797 in the promoter (Fig. 5A). We examined the requirement of this sequence element for the prolactin-stimulated induction of PIII in two experiments. First, we deleted a 51 bp segment containing the STAT site from the reporter gene construct \( (-2999) \). Expression of the resulting clone (clone Del, Fig. 5B) was no longer induced by prolactin alone \( (0·9 ± 0·07\text{-fold}; n=3) \) or by the combined action of prolactin and dexamethasone \( (0·9 ± 0·14\text{-fold}) \). It retained some inducibility by dexamethasone alone \( (1·8 ± 0·32\text{-fold}) \). Secondly, we altered the consensus STAT

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**Figure 4** PIII is primarily expressed in MECs. In situ hybridizations of antisense (probe) or sense (control) probes towards two different areas of a pregnant (a, c) and lactating (b, d) mammary glands of cows. The probe was derived from a subclone containing only exon 5A as insert and labeled with \( ^{35}\text{S}\text{ATP} \). Slides were developed after 2·5 months exposure, counterstained with Gill’s rapid hematoxylin and eosin. Arrows exemplify alveoli decorated with silver grains (solid arrows) or areas of adipocytes devoid of silver grains (dashed arrows). Low magnification overviews (a and c: ×100) and a higher magnification overviews (b and d: ×200) are shown.
Two point mutations were introduced into clone –2999 changing the consensus STAT-binding sequence from TTCNNNGAA to TT TNNNGT A.

The inducibility of the resulting clone (M1, Fig. 5B) by either of the hormones was greatly diminished when compared with the original clone (1·2-fold for prolactin and 1·5-fold dexamethasone in the mutant vs 2·4-fold for prolactin and 3·8-fold for dexamethasone in the wild-type clone). The combined action of both hormones induces clone M1 only 1·5-fold, compared with 4·0-fold for the wild-type clone, (n=6). Thus, inducibility of PIII by prolactin and dexamethasone in HC11 cells requires integrity of the consensus sequence for STAT factor binding.

**STAT5 binds to the STAT site at –797 of PIII in the lactating mammary gland**

Having established the importance of the STAT factor binding site for lactogenic induction of PIII, we examined which of the different STAT factors in the lactating mammary gland of the cow actually interacts with this site. Electrophoretic mobility shifts assays (EMSA) were performed using nuclear extracts from lactating mammary gland tissue and an oligonucleotide containing the putative STAT binding site. A DNA–protein complex was detected (Fig. 6) which could be competed away with a 100- or 50-fold molar excess of the unlabeled probe. It remained slightly visible if the unlabeled competitor was in only a 25-fold molar excess of the probe. The shifted complex remained unaffected if a mutated oligonucleotide was used as competitor harboring the mutation M1 of the STAT-binding site (cf. Fig. 5). This result shows that the proteins binding to the probe address the core sequence of the STAT factor binding site. The shifted band is entirely supershifted by an antibody known to be monospecific for STAT5A (Fig. 6, lane α5A). This identifies STAT5A as protein binding to this sequence motif of PIII in the lactating mammary gland of the cow.

**Discussion**

We characterize for the first time the bovine PIII of the ACC-α-encoding gene, known to be rate limiting for fatty acid synthesis. We show that PIII-derived transcripts are expressed at high levels exclusively in bovine MECs during lactation, and that prolactin stimulates expression of PIII in transfected mammary cells. The data provide the first direct evidence for the involvement of STAT5 recognition sequence by site directed mutagenesis.
factors in the regulation of a gene involved in fatty acid metabolism.

The relevance of PIII-derived transcripts for milk fat production

A number of observations suggest that the biological role of PIII is to induce milk fat production during lactation. Firstly, PIII is the only one of the three ACC-α-expressing promoters to be strongly induced in the mammary gland at lactation. In contrast, the fraction of PII-derived transcripts from the total number of ACC-α-encoding transcripts remains virtually constant at ~33% in pubertal and lactating mammary glands (Mao et al. 2001), and the level of PII-derived transcripts also appears to be relatively unaltered during lactation in the bovine mammary gland, as judged from the RNase protection assays (Fig. 3B) and real time-PCR quantification data (J Mao & H-M Seyfert 2002). Secondly, our in situ hybridizations show that the greatly enhanced expression of PIII in the lactating mammary gland occurs almost exclusively in the MECs of the mammary gland.

Figure 6 STAT5 molecules are recruited into the mammary gland-specific complex of the EMSA-probe for PIII. The 32P-labeled probe was incubated with nuclear extracts of mammary tissue from a lactating cow. The probe forms a specific complex (arrow s, lane -) for which the unlabeled wild-type probe (wt) competes, but not the mutated oligonucleotide M1 (M1). The molar fold excess of the competitors is indicated. The shifted band is entirely supershifted (arrow ss) if 1 µg of the monospecific antibody against STAT5A is added (lane α5A). Lane FP contained only the probe, to which the same amount of antibody had been added in the sample resolved in lane FP α5A.
MECs are the site of milk fat production (Rohlfs et al. 1993, Neville & Picciano 1997). Therefore, our data together propose a significant role for the PIII-derived enzymatic isoform of ACC-α for milk fat production. However, verification of this requires further investigation. PIII-derived transcripts may comprise only ~8% of the total ACC-α mRNA molecules in the lactating gland. Thus, the roles of the other two promoters for milk fat production need further examination. Expression of PIII may be peculiar to the Bovidae species complexes, since (i) it has not been described in other species and (ii) we failed to amplify PIII-derived transcripts from mouse mammary glands in 5’-RACE experiments, despite considerable efforts. The significance of this apparent species restriction in utilization of PIII is unknown.

Prolactin–STAT5 signaling is crucial for lactogenic-induced ACC-α expression in MECs

It was somewhat unexpected to realize in pilot experiments that our PIII expression construct could be induced with prolactin and dexamethasone in the murine MEC line HC11, since PIII is apparently not expressed in vivo in mice. Prolactin signals are mediated by STAT5 (Rosen et al. 1999) and there is some evidence that STAT-factors might be important for regulation of fat metabolism. STAT3 mediates the leptin signal (Vaisse et al. 1998). Therefore, STAT5 factors having bound to their target complexes, since (i) it has not been described in other species and (ii) we failed to amplify PIII-derived transcripts from mouse mammary glands in 5’-RACE experiments, despite considerable efforts. The significance of this apparent species restriction in utilization of PIII is unknown.

Biological implications for the balanced synthesis of milk components

The interaction of STAT5 with PIII in the mammary gland provides the first evidence for the assumption that prolactin might have a pivotal role in stimulating fatty acid synthesis and, by inference, milk fat synthesis during lactation. Lactogenesis induces the expression of PIII in the mammary epithelial cells and this requires the activity of prolactin-activated factors which are also known to be highly relevant for the expression of milk protein.
genes. The very specific role of prolactin in the regulation of gene expression in MECs is reinforced by the recent demonstration that, within the mammary gland, STAT5 is activated by different hormones in different compartments, but that prolactin selectively activates STAT5 in the mammary epithelium (Gallego et al. 2001). Thus, prolactin signaling as mediated via STAT5 is not only highly relevant for regulating milk protein synthesis, but may also represent a crucial determinant for milk fat synthesis. Our data suggest that part of the genetic basis for the inherited balanced milk protein vs milk fat ratio will be found among components of the prolactin–STAT5 pathway of signal transduction operating in mammary epithelial cells.

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