

Changes in DNA-binding activity of transcription factors in the differentiating bovine mammary gland

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Our earlier studies carried out *in silico* demonstrated that different transcription factors have their putative binding sites in the 5'-flanking regions of bovine milk protein genes. Now we extended our study to include the experimental analysis of these transcription factors. This study on electrophoretic mobility shift assay (EMSA) of nuclear proteins derived from bovine mammary glands showed for the first time the presence there of CREB, NF- κ B, Oct1 and Sp1 transcription factors. The pattern of DNA-protein complexes of Sp1 transcription factor significantly differed between virgin heifers and lactating cows. Transition from pregnancy to lactation was associated with changes in the DNA-protein binding patterns of NFI and Oct1 transcription factors.

KEY WORDS: cattle / casein genes / DNA-binding / transcription factor

One of the most interesting and important problems in molecular biology is mechanism underlying in tissue- and stage-specific gene expression. Control of gene expression at the transcriptional level requires interactions of transcription factors and their binding sites in a gene promoter or enhancer. Mammary gland epithelial cells are highly specialized for the synthesis of limited number of proteins during lactation and involution. Most of the mRNAs expressed in the bovine mammary gland during lactation encode caseins and whey proteins, which account for approximately 95% of milk proteins [Karal-Albrecht *et al.* 2000]. Regulation of milk protein genes has com-

mon features: all of them are expressed only in mammary gland epithelium during late pregnancy and lactation, their transcription is induced by lactogenic hormones – insulin, prolactin and glucocorticoids, while repressed by progesterone. These features make mammary gland a good model for studying mechanisms of tissue- and stage-specific gene expression.

The main step on which regulation of gene expression occurs is the initiation of transcription. Minimum requirement for the initiation of gene transcription is binding of an appropriate set of transcription factors (TFs) to its 5'-upstream region. The combination of factors is determined by the regulatory sequences of a gene and each transcription factor recognizes DNA sequence to which it binds. Computer analysis of cows' milk protein gene promoters had shown potential binding sequences for various TFs [Malewski and Zwierzchowski 1995, Malewski 1998]. Their 5'-flanking regions contain putative binding sites for mammary gland-specific – MAF, STAT5, PMF, as well as ubiquitous TFs – AP1, CREB, GR, NFI, NF-kB, Oct1, Sp1, YY1 [Malewski and Zwierzchowski 2002].

In the last several years the regulation of expression of milk protein genes was extensively studied, and the number of TFs involved in their induction has been characterized. These factors include C/EBP, CTF/NFI, GR, MAF, PMF, STAT5, YY1 [Rosen *et al.* 1996, 1999]. Dairy cattle are the main milk producers. Despite the unquestioned economical value of milk, molecular mechanisms regulating expression of the milk protein genes in cattle are poorly understood. Recently, in the cow mammary gland were studied only STAT [Yang *et al.* 2000, Wheeler *et al.* 2001], peroxisome proliferator-activated receptors [Sundvold *et al.* 1997] and NFI [Ivanov *et al.* 1990] transcription factors.

The aim of the current study was the analysis of DNA-binding activity of those TFs of which putative binding sites are present at bovine milk protein gene 5'-flanking sequences. Changes of TFs in the mammary gland during pregnancy, lactation and involution were analysed by the electrophoretic mobility shift assay (EMSA). The specificity of DNA-protein complexes was analysed by titration with increasing amounts of appropriate unlabelled oligonucleotides.

Material and methods

Animals and tissues

Mammary glands were used of Holstein-Friesian heifers and cows at different physiological stages: two virgin heifers; two pregnant heifers; two lactating cows (month 2 of first lactation) and two cows with involuting mammary gland (week 2 after drying-off). Samples were excised immediately *post mortem* from udders of the animal slaughtered at the local abattoirs. Mammary tissue was cleared from adjacent tissues, frozen at -25°C and stored at -75°C until use.

Preparation of nuclei

Nuclei were prepared from frozen tissues according to Wakao *et al.* [1992]. Equal samples of mammary tissues derived from 3-4 cows at the same physiological status were pooled and used for preparation of cell nuclei. Mammary gland tissues were homogenized in 10 volumes of buffer: 10.0 mM HEPES-NaOH pH 7.5; 10.0 mM NaCl; 0.1 mM EDTA; 0.1 mM EGTA; 1.0 mM DTT, 0.7 mM spermidine; 0.15 mM spermine; 0.2 mM PMSF and 0.1% NP-40) in a Polytron type homogenizer (KINEMATICA). The NaCl concentration was raised to 0.1 M by the addition of an appropriate volume of 5.0 M NaCl. After filtration through nylon nets, the homogenate was centrifuged at 1,800 g for 10 min. The nuclei were then washed once with buffer and pelleted by centrifugation.

Extraction of whole nuclear proteins

Nuclei were suspended (0.125 v/w of the original tissue) in the buffer: 20.0 mM HEPES-NaOH pH 7.5; 600.0 mM NaCl; 0.2 mM EDTA; 2.0 mM EGTA; 1.0 mM DTT; 0.7 mM spermidine; 0.15 mM spermine; 0.1% NP-40; 0.2 mM PMSF; 25% (v/v) glycerol and incubated on ice for 40 min with gentle agitation. Then the nuclei were removed by centrifugation at 40,000 g for 20 min. and the nuclear proteins were dialysed against 10 v of buffer: 20.0 mM HEPES-NaOH pH 7.5; 50.0 mM NaCl; 2.0 mM EDTA; 1.0 mM DTT, 0.2 mM PMSF; 0.1% NP-40; 10% glycerol. The precipitate formed during the dialysis was removed by centrifugation at 40,000 g for 30 min. Extracts of nuclear proteins were divided into portions and stored at -75°C.

Preparation of probes

For measuring CREB, CTF/NFI, NF-κB, Oct1 and Sp1 DNA-binding activities oligonucleotides from the Transcription Factors Bandshift Kit (Gibco-BRL) were used (consensus sequences bolded and underlined):

CRE 5'-GATCTGACGTCATGACTGACGTCATGACTGACGTCATCA-3'

NFI 5'-GATCGCCAATGATCGCCAATGATCGCCAATGATC-3'

NF-κB 5'-GATCCAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3'

Oct1 5'-GATCATGCAAATGATCATGCAAATGATCATGCAAAT-3'

Sp1 5'-ATCGGGGCGGGGATCGGGGCGGGGATCGGGGCGGGG-3'

Complementary oligonucleotides were annealed at room temperature. For labelling of the probes 2 pmoles of the double-stranded oligonucleotides with extended 5' ends were elongated with 6.0 U of Klenov fragment of DNA polymerase I, 1.0 mM of each of dATP, dGTP, dTTP and 50.0 mCi (1.85 MBq) of [α -³²P] dCTP (ICN). The labelled probes were purified from unbound nucleotides using QIAquick Nucleotide Removal

Kit (QIAGEN).

Electrophoretic mobility shift assay (EMSA)

EMSA analyses were performed according to Schmitt-Ney *et al.* [1991]. Twenty femtomoles (~50,000 cpm) of a labelled probe were incubated with 5 µg of nuclear proteins for 30 min. at room temperature in a 20-µl final reaction in buffer recommended by SantaCruz Biotechnology, Inc. (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1.0 mM DTT, 1.0 mM EDTA and 5% glycerol). Then, 2 µl of loading buffer (50% glycerol, 0.25% bromphenol blue) was added and the reaction mixture was loaded on a prerun 6% non-denaturing polyacrylamide gels in 0.25× Tris-borate-EDTA (TBE). The electrophoresis was performed at room temperature at 100 V until the bromphenol blue had migrated to the end of gel. All EMSA analyses were performed twice.

The gels were dried in a BioRad (GEL DRYER 543) apparatus and subjected to radioautography with Kodak Screen. Autoradiograms were scanned with Bio-Rad Molecular Imager FX and densitometry was made with Quantity One programme (Bio-Rad).

Results and discussion

CRE DNA-binding activity

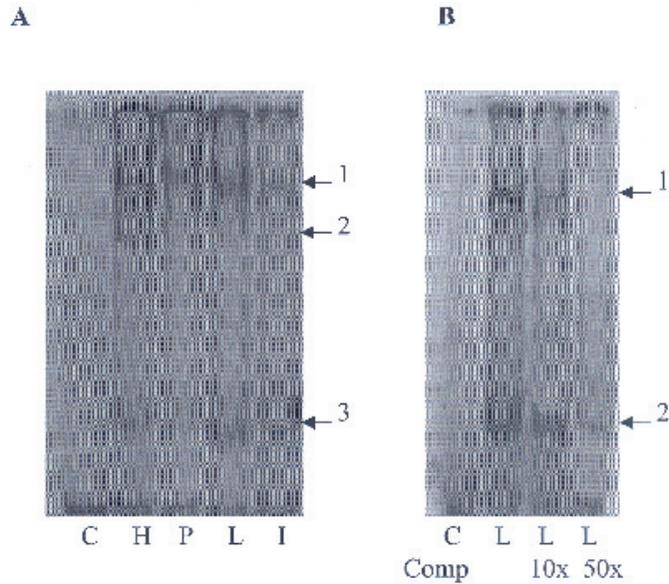
Crude nuclear protein extracts from the mammary glands form three DNA-protein complexes with the CRE probe (Photo 1A). Complex 2 is specific for virgin heifers; two other complexes (1 and 3) are present at all investigated stages of mammary gland, but most abundant during lactation. Drying-off was associated with sharp decrease of both (1 and 3) DNA-protein complexes. The specificity of DNA-protein binding was estimated by competition with 10- and 50-fold excess of unlabelled probe. All the DNA-protein complexes appeared specific for the DNA, 50-fold excess competitor almost completely eliminated the binding of proteins to DNA (Photo 1B; for complex 2 data not shown).

NFI DNA-binding activity

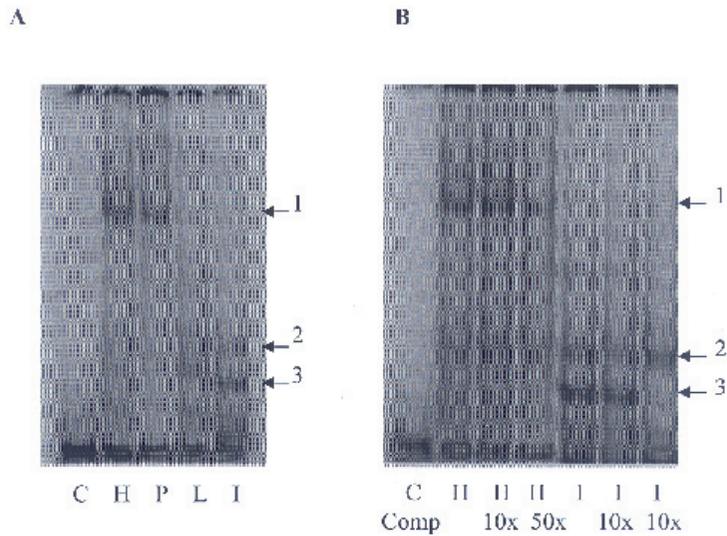
In the extracts of nuclear proteins derived from the mammary glands of virgin and pregnant heifers a single, slow-migrating NFI DNA-protein complex was found (Photo 2A). Transition to lactation was associated with disappearance of slow-migrating (1) and appearance of two fast-migrating complexes (2 and 3). Competition experiments showed that complex 2 formed by proteins from involuting mammary gland of dried-off cows was non-specific, while both 1 and 3 complexes were the sequence-specific (Photo 2B).

NF-κB DNA-binding activity

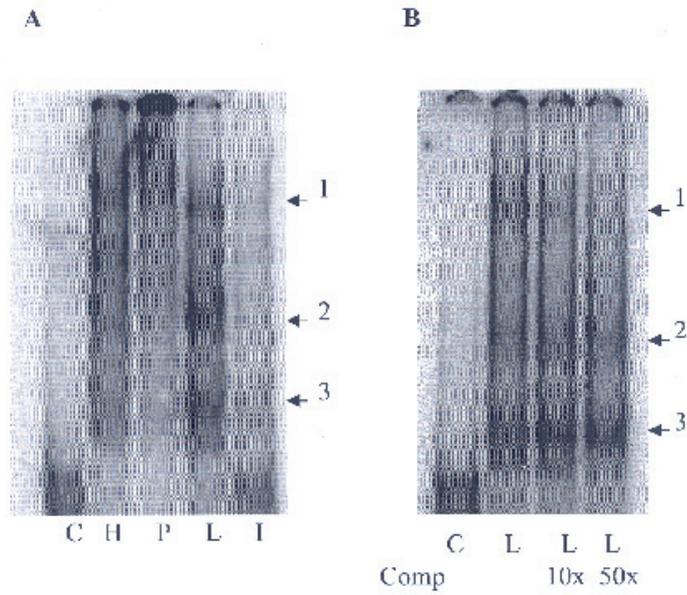
DNA-binding activity of transcription factors in the bovine mammary gland



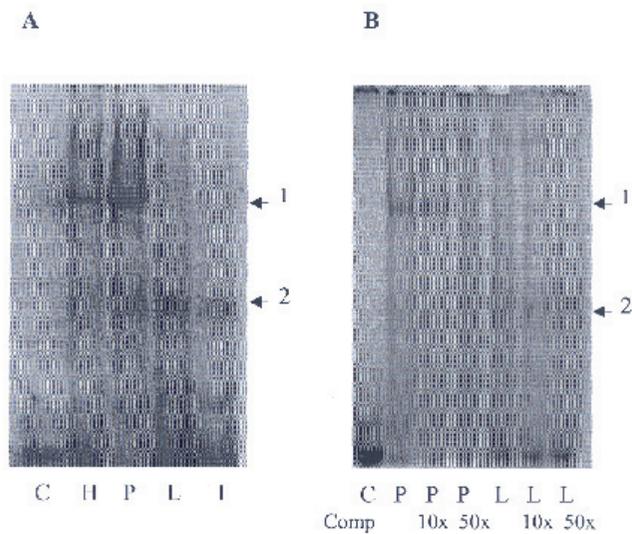
Photos 1A and 1B. CREB DNA-binding activity in the bovine mammary gland nuclear extracts. **A:** nuclear proteins derived from virgin heifers (H), pregnant heifers (P), lactating cows (L) and involuting cows (I) analysed with EMSA with labelled CRE probe. C – control (free probe). Arrows indicate DNA-protein complexes. **B:** competition analysis – unlabelled CRE probe was used as a competitor in 10× or 50× excess.



Photos 2A and 2B. NFI DNA-binding activity in the bovine mammary gland nuclear extracts. **A:** nuclear proteins analysed with EMSA with labelled NFI probe. **B:** competition analysis with unlabelled NFI probe. Other details as in the legend to Photos 1A and 1B.



Photos 3A and 3B. NF- κ B DNA-binding activity in the bovine mammary gland nuclear extracts. **A**: nuclear proteins were analysed with EMSA using labelled NF- κ B probe. **B**: competition analysis with unlabelled NF- κ B probe. Other details as in the legend to Photos 1A and 1B.



Photos 4A and 4B. Oct1 DNA-binding activity in the bovine mammary gland nuclear extracts. **A**: nuclear proteins analysed with EMSA with labelled Oct1 probe. **B**: competition analysis with unlabelled Oct1 probe. Other details as in the legend to Photos 1A and 1B.

The NF- κ B DNA-binding activity was found in the mammary gland nuclear extracts of virgin and pregnant heifers, and of lactating cows (Photo 3A). However competition experiments showed that only one of them, *i.e.* the DNA-protein complex 1 was NF- κ B-specific (Photo 3B).

Oct1 DNA-binding activity

In the extracts of nuclear proteins derived from the mammary glands of virgin and pregnant heifers two DNA-protein complexes with the Oct1 probe were found (Photo 4A). Competition experiments showed that complexes formed by proteins from lactation and involution stages were non-specific, while that formed in pregnant and virgin heifers was sequence-specific (Photo 4B). It was shown that the Oct1 DNA-binding activity strongly increased at pregnancy and was completely absent during lactation.

Sp1 DNA-binding activity

Sp1 binding to DNA was found in the mammary gland nuclear extracts at all investigated physiological statuses, but the pattern of DNA-protein complexes was specific for each status. Nuclear proteins from the virgin heifer's mammary gland formed DNA-protein complexes 1, 2, 3 and 4 (Photo 5A). Pregnancy was associated with increased amount of DNA-protein complex 1, and decreased amount of complex 2. Transition to lactation appeared to be related to sharp decrease of complex 1 and

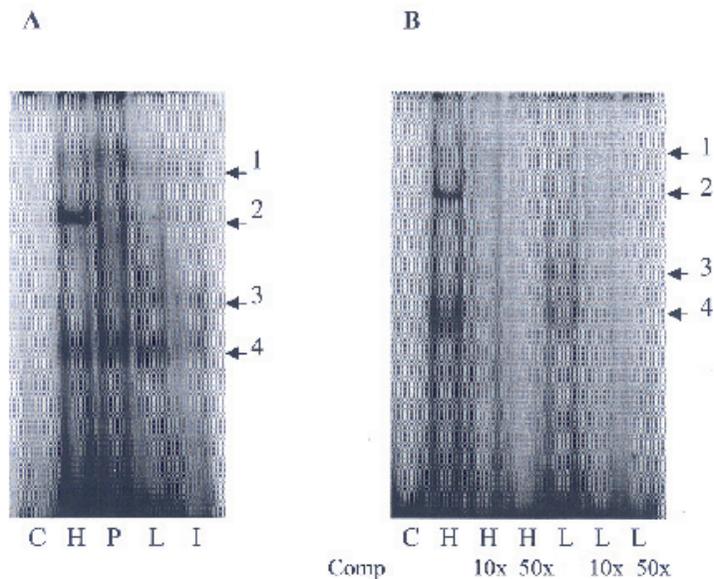


Photo 5A and 5B. Sp1 DNA-binding activity in the bovine mammary gland nuclear extracts. **A:** nuclear proteins analysed with EMSA with labelled Sp1 probe. **B:** competition analysis with unlabelled Sp1 probe. Other details as in the legend to Photos 1A and 1B.

appearance of new complex 3. During involution of mammary gland the amount of all DNA-protein complexes strongly decreased. Specificity of Sp1 DNA-protein complexes was confirmed by competition experiments (Photo 5B).

In the last several years the transcription factors were extensively studied in the mammary glands of mice and rats, and certain transcription factors involved in the induction of the milk protein genes have been characterized. However, very little is known about transcription factors in the bovine mammary gland. Investigation of biological role of STAT5 showed significant differences in controlling lactation between rodents and ruminants [Wheeler *et al.* 2001].

NFI proteins were shown to be important for the regulation of gene expression in many tissues [Gronostajski 2000]. They are also important for the expression of WAP [Li and Rosen 1995] and β -lactoglobulin genes [Watson *et al.* 1991]. Mukhopadhyay *et al.* [2001] used RNase protection assay to study expression of NFI genes (A, B, C, and X) in the mouse. NFI-A expression at the transcription level was fairly constant at different stages of mammary gland development. Expressions of NFI-B and NFI-X genes were at the highest level during lactation and then decreased during involution. In contrast, NFI-C expression decreased significantly during lactation and increased during involution [Mukhopadhyay *et al.* 2001]. EMSA experiments of Kannius-Janson *et al.* [2002] showed that the level of NFI DNA-protein complex sharply increases on day 13 of pregnancy and is maintained at this level till day 1 of lactation but is reduced 2 days after weaning.

This study showed that nuclear proteins from the bovine mammary gland form two NFI-specific DNA-protein complexes (Photo 2A). Changes of slowly migrating complex 1 show some similarity to NFI DNA-protein complex in mice. This DNA-protein complex was abundant in virgin and pregnant heifers but sharply decreased during lactation. Lactation is associated with appearance of fast migrating DNA-protein complex 2 probably earlier described by Ivanov *et al.* [1990]. Amount of complex 2 increases during mammary gland involution. These differences in activity of NFI between murine and bovine mammary gland could be due to the fact that in the latter pregnancy, lactation and involution are not sequential but overlapping events and at the same time cow could be pregnant and lactating or pregnant and involuting. Information about DNA-protein complexes could be more comprehensive if purified transcription factors and antibodies to transcription factors would be available. Unfortunately, purified transcription factors and antibodies are yet not available for cattle.

Immunohistochemistry analysis of NF- κ B revealed expression of the p105/p50 and RelA subunits in the epithelium of virgin, pregnant, lactating, and involuting mammary gland of mouse [Brantley *et al.* 2000]. Expression was observed in both the epithelium and stroma. At pregnancy, when the mammary gland epithelium proliferates and begins to express milk proteins, RelA expression is upregulated. EMSA experiments by Clarkson and Watson [1999] demonstrated that DNA-binding complexes containing p50 and RelA were abundant during pregnancy and involution, but not during lactation.

As shown by Geymayer and Doppler [2000] the Sp1 binding activity was detected in the mammary gland of virgin mice and its activity increased during pregnancy, and remained on the same level until day 10 of lactation. In nuclear extracts from involuting mammary gland Sp1 activity was not found. In the bovine mammary gland we observed significant changes in pattern of DNA-protein complexes during pregnancy, lactation and involution (dry period).

The results presented here strongly suggest that CREB, NFI, NF- κ B, Oct1 and Sp1 transcription factors are present in the bovine mammary gland and are expressed at different levels during the lactation-reproduction cycle. Pregnancy is associated with changes in Sp1 DNA-protein binding activity. During transition from pregnancy to lactation take place changes of pattern of DNA-protein binding activity of transcription factors NFI, Oct1 and Sp1.

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Zmiany aktywności wiązania czynników transkrypcyjnych z DNA w różnych stadiach fizjologicznych gruczołu mlekowego bydła

Streszczenie

Weześniejsze badania autorów prowadzone *in silico* wykazały obecność potencjalnych miejsc wiązania dla wielu czynników transkrypcyjnych w rejonach 5'-flankujących genów białek mleka. W obecnej pracy przedstawiono wyniki analizy poszerzonej o doświadczalne badanie wiązania tych czynników transkrypcyjnych z DNA. Stosując analizę EMSA z użyciem białek jądrowych gruczołu mlekowego bydła w różnych stadiach jego rozwoju, po raz pierwszy wykazano obecność w nim czynników transkrypcyjnych CREB, NF- κ B, Oct1 i Sp1. Kompleksy DNA-białko czynnika transkrypcyjnego Sp1 wykazały różne wzory ruchliwości elektroforetycznej u dziewiczych i cielnych jałówek. Przejście od ciąży do laktacji wiązało się z dalszymi zmianami w kompleksach DNA-białko czynników transkrypcyjnych NFI i Oct1.